Childhood Leukemia: Origins and Biomarkers

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Executive Summary

Leukemia, the most common type of pediatric cancer and a leading cause of disease-related death among children, represents a heterogenous group of clonal hematopoietic cell disorders. These are classified into distinct types and subtypes by morphologic immunologic and genetic features. Acute lymphoblastic leukemia and acute myeloid leukemia are the two most common types affecting children, and the incidence of both has been increasing in recent years in the United States and other developed countries. The reasons for the increasing incidence are unknown but are commonly believed to be related to development and improved socioeconomic conditions.

Current evidence indicates that most leukemias originate through a multistage process involving a variable series of genetic alterations. For most ALL and infant AML, the initial genetic change is a translocation or other abnormality that originates prior to the birth of the child. A second critical genetic event occurs postnatally that triggers the onset of the disease. However, exposure to potent leukemia-inducing agents either prenatally or postnatally can be sufficient by itself to induce leukemia in an exposed child. Ionizing radiation, various alkylating agents and topoisomerase II inhibitors have all been shown to cause leukemia in children. While conclusive evidence on other environmental and industrial agents is currently unavailable, it would appear likely that agents that induce leukemia in adults would also be capable of inducing leukemia in children.

In recent years, a large number of biological markers (biomarkers) have been developed that are providing insights into the origins and progression of leukemia, and are allowing the identification of individuals who are at increased risk for developing the disease. These biological markers are often classified into three general categories – biomarkers of exposure, effect, and susceptibility. Extensive efforts have been made over the past 20+ years to develop and validate biomarkers in each of these categories for many of the known and suspected leukemia-inducing agents. In this report, numerous examples of the various types of biomarkers are provided, as well as illustrations of their application for monitoring human exposures to leukemia-inducing agents. Of particular interest is the development of leukemia-related genetic biomarkers that is providing new insights into the progression of the disease, and is allowing individuals at particularly

high risk for developing leukemia to be monitored. In addition, an increasing number of leukemia-related genetic polymorphisms and related risk factors are being identified. While significant progress has been made, the development of effective prospective biomarkers continues to be challenging, due in part to the heterogeneity of the disease combined with its rarity and variable onset. It is hoped that continuing advances in genomic, proteomic and computational technologies as well as the development of new leukemia models using transgenic animals will provide additional tools that will improve future biomarker studies and facilitate the identification of at-risk children and adults.

Introduction

Overall incidence and trends

While uncommon, childhood leukemia still accounts for approximately 30% of all childhood cancers in the United States, and is a leading cause of disease-related death among children (Smith *et al.*, 2005a; ACS, 2006). The incidence of leukemia in children (36 per million) is similar to that seen in young to middle-aged adults (ages 20-44) but roughly one-tenth of that of adults aged 45 years and older, where the annual incidences increase with age from 144 to 545 per million (Xie *et al.*, 2003; ACS, 2006). Moreover, the incidence trends for adult and pediatric leukemias differ substantially. While the overall trend for adult leukemia has declined, the incidence of childhood leukemias has increased noticeably in recent years, largely due to the prevalence of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) in children which have increased by 1.1% and 0.5% per year, respectively (Xie *et al.*, 2003). Similar increases have been seen across Europe and in other developed countries (Hrusak *et al.*, 2002; Steliarova-Foucher *et al.*, 2004). Fortunately, there has been significant progress in treating childhood leukemias so that the five-year survival rate for the affected children is now approximately 80% (ACS, 2006).

Description and Heterogeneity

Leukemia represents a group of hematopoietic cell disorders characterized by an uncontrolled proliferation or expansion of cells that do not develop or retain the capacity to differentiate normally and form mature blood cells (Sawyers *et al.*, 1991). These

neoplasms represent clonal expansions of hematopoietic cells, almost always within either the myeloid or lymphoid lineage (Nowell, 1991). Infrequently, some leukemias exhibit both myeloid and lymphoid characteristics and are known as biphenotypic leukemias (Russell, 1997). Both myeloid and lymphoid clones are designated as chronic or acute leukemia, depending on the rate of clonal expansion and the stage of differentiation that dominates the leukemic clone (Nowell, 1991). Acute leukemias tend to have a rapid onset with a predominance of immature cells, whereas chronic leukemias have a more insidious onset and progress over a period of months or years to a blast or acute leukemic phase.

Using this basic classification, leukemias can be described as one of four major types – ALL, AML, chronic lymphoblastic leukemia (CLL), and chronic myeloid leukemia (CML). Within these larger groupings, there are numerous subtypes that have unique characteristics, origins, and increasingly recognized, clinical significance. These sub-types are generally classified according to morphologic, cytogenetic, immunophenotypic and more recently, molecular characteristics according to the French-American-British (FAB) or World Health Organization classification systems (Head and Pui, 1999; WHO, 2001; Haferlach *et al.*, 2005). The two major diagnostic categories, ALL and AML, can be further classified based upon cellular features. Childhood ALL is subdivided by FAB morphology (L1, L2, and L3) and by immunophenotype (B-cell, early pre-B, pre-B, and T cell) (Bhatia *et al.*, 1999). Childhood AML is classified primarily by morphological characteristics into eight different subgroups (M0-M7) based upon the degree of maturation and myeloid lineage involved. Similarly, the myelodysplastic syndromes (MDS) are a series of blood disorders characterized by maturation defects resulting in ineffective hematopoiesis. These syndromes, also classified by the FAB and WHO systems, are commonly considered as preleukemic because of a variable, but significant, proportion (1 to 33%) of the various disorders progress to frank leukemia (Wright, 1995; WHO, 2001; Hasle *et al.*, 2003). An outline of the primary types and subtypes of lymphohematopoietic diseases as classified by the WHO is shown in Table 1. For convenience, a simplified classification scheme based largely on the FAB classification system is outlined in Table 2.

ALL represents the predominant form of leukemia in children accounting for approximately 75% of all cases (Bhatia and Robison, 2003). Of these, about 80% express cell surface markers indicative of a precursor B-cell lineage, 15-20% exhibit T-cell markers, and 1 to 2% express a mature B cell phenotype (Esparza and Sakamoto, 2005). Of the remaining childhood leukemias, AML accounts for 15 to 20%, CML for 5%, and CLL is only rarely seen in children. The age-incidence rate for both ALL and AML among children is highest for those younger than 5 years of age but differs substantially for these two major types of leukemia. In the United States and other developed countries, the age-incidence curve for ALL is characterized by a peak between the ages of 1 and 4 that is most pronounced among 2 to 3 year olds with a peak incidence (80 per million) that is four-fold higher than that of infants and nearly 10-fold higher than that of 19 year olds (Bhatia and Robison, 2003). This unusual distribution is observed in other countries and has been attributed to factors associated with development and increasing socioeconomic conditions (Hrusak *et al.*, 2002; Steliarova-Foucher *et al.*, 2004). The observed peak in ALL has been reported to originate in the B-precursor cells, and genetically is believed to have the TEL/AML1 or hyperdiploid genotype (Sullivan, 1993; Hrusak *et al.*, 2002).

In contrast, the age-incidence rates for AML are highest in infancy (~10 per million), then decline after the first year of life, and become fairly similar for older children (5-6 per million) (Bhatia and Robison, 2003). For CML, the overall incidence is much lower and the distribution is fairly uniform with age, ranging from 1.6 per million in children aged 1 to 4, to 1.2 per million in infants and 0.7 per million for those aged 10 to 14 (Bhatia and Robison, 2003).

Origins of leukemia

Acute leukemia is a clonal disorder of the hematopoietic system, arising in a single cell through a series of mutations that is passed on to its progeny cells. In most cases, the genetic alterations are acquired, with <5% associated with inherited genetic syndromes (Bhatia *et al.*, 1999). For example, 2.6% of children with leukemia in one British study were reported to have a recognized genetic condition, with Down syndrome children accounting for almost all of those observed (2.3%) (Bhatia and Robison, 2003).

In addition, there are a number of normal inherited polymorphisms typically in genes involved in xenobiotic metabolism, DNA synthesis or repair that may contribute indirectly to the risk of leukemia. The origin of childhood leukemia as well as major types of risk factors are described in more detail below.

Genetic alterations

In addition to morphologic and immunophenotypic variability, adult and childhood leukemias also exhibit extensive genetic heterogeneity. The common clonal cytogenetic changes include alterations in chromosome number such as loss or gain of one or more chromosomes (e.g monosomy 7 and trisomy 8, respectively), balanced and unbalanced chromosome translocations, deletions or inversions involving specific chromosomal regions as well as complex arrangements involving combinations of the above. In some cases, these are a reflection of the genetic instability that is common in many types of cancers. However in many cases, these are highly specific involving genes that are directly involved in the cancer process. For example, the translocation between chromosomes 15 and 17 {t(15;17)(q22;q12-21)} that is characteristic of the promyelocytic form of AML (FAB M3) results in a fusion gene involving the PML gene on chromosome 15 and the retinoic acid receptor alpha (RARa) gene on chromosome 17. This hybrid gene blocks differentiation of the developing myeloid cells at the promyelocytic stage (Downing, 1999), a characteristic feature of the disease. Listings of recurrent chromosomal changes seen in childhood ALL and AML are shown in Tables 3 and 4. As apparent from these Tables, there is considerable genetic heterogeneity in both lymphocytic and myeloid leukemias. All of the major cytogenetically abnormal subtypes of leukemia present in children are also present in adults although the incidences in the two groups may vary.

In addition to these microscopically visible chromosomal alterations, mutations at the molecular level affecting specific cancer-related genes such as *RAS*, *FTL3*, *GATA1,* and *TP53* have also been seen in leukemias at varying frequencies. For example, mutations in genes of the receptor tyrosine kinase/RAS-BRAF signal transduction pathway are reported to be present in over 50% of de novo AML (Christiansen *et al.*, 2005). These occur through many mechanisms including base pair substitutions, frame

shifts, internal tandem duplications, gene fusions and splicing errors {for examples in *AML1*, see (Roumier *et al.*, 2003)}. In addition, changes in methylation patterns in leukemia-related genes or their promoter regions have also been seen in pediatric leukemias (Zheng *et al.*, 2004). Examples of genes altered by mutation in various types of childhood leukemias are shown in Table 5. To date, over 300 different genetic alterations and mutations have been identified (Kelly and Gilliland, 2002). Indeed, a recent examination of alterations affecting the *MLL* gene, a cancer-related gene located at band 11q23, in pediatric and adult leukemias identified a total of 87 different rearrangements, primarily translocations, involving this one gene (Meyer *et al.*, 2006). While most of the detected alterations are rare, certain translocations and genes are more prevalent, and are typically associated with specific leukemic subtypes (Bhatia *et al.*, 1999; Greaves and Wiemels, 2003). Of particular note in children, are translocations involving the *MLL* gene which are found in approximately 85% of infant ALL and 50% of infant AML, and the t(12;21)(p13;q22) which forms the TEL-AML1 fusion gene that is present in ~20% of B-cell precursor ALL (Greaves and Wiemels, 2003). Hyperdiploidy is also present in approximately 35% of B-cell precursor ALL.

In recent years, researchers have begun to identify patterns among the myriad of translocations, gene arrangements, and point mutations involved in myeloid leukemias and have created a model for leukemogenesis (Deguchi and Gilliland, 2002; Kelly and Gilliland, 2002). According to the current model proposed by (Kelly and Gilliland, 2002), "AML is the consequence of a collaboration between at least two broad classes of mutations. Class I mutations, exemplified by constitutively activated tyrosine kinases or their down stream effectors, such as *BCR/ABL*, *TEL/PDGFβR*, *N-RAS*, or *K-RAS* mutants, or constitutively activated *FLT3*, confer a proliferative or survival advantage to hematopoietic cells. When expressed alone, these mutant genes confer a CML-like disease characterized by leukocytosis with normal maturation and function of cells. Class II mutations result in loss of function of transcription factors that are important for normal hematopoietic differentiation and include the *AML1/ETO*, *CBFβ/SMMHC*, *PML/RARα*, and *NUP98/HOXA9* fusions as well as point mutations in hematopoietic transcription factors such as *AML1* and *C/EBPα*. These mutations would also be predicted to impair subsequent apoptosis in cells that do not undergo terminal

differentiation. When expressed alone, these mutations may confer a phenotype like most MDS. Regardless of the timing or order of acquisition of mutations, individuals who accrue both Class I and Class II mutations have a clinical phenotype of AML characterized by a proliferative and/or survival advantage to cells and by impaired hematopoietic differentiation." At this time, a similar unifying model has not been developed for lymphocytic leukemias. However, given the involvement of genes common to both ALL and AML, a model for lymphoid leukemogenesis would be expected to involve as least some parts of the same model. A more detailed model which will be presented in later section, has also been proposed by Pedersen-Bjergaard for therapy-related MDS and AML (Pedersen-Bjergaard *et al.*, 1995; Pedersen-Bjergaard *et al.*, 2002).

Inherited genetic and familial risk factors:

A number of genetic syndromes have been shown to be associated with an increased risk of leukemia, most cases of which are observed in children (Bhatia and Robison, 2003). These include syndromes involving DNA repair deficiences such as ataxia telangiectasia, Bloom syndrome, and Fanconi anemia, defects leading to second mutations such as Schwachman syndrome, familial platelet disorder, amegakaryocytic thrombocytopenia, various tumor suppressor-related defects such as Down syndrome, Li-Fraumeni and neurofibromatosis type 1 as well as a number with currently unidentified origins or functions (Segel and Lichtman, 2004). Examples of inherited syndromes predisposing children to leukemia are shown in Table 6. In spite of the large number of syndromes, only a very small proportion of leukemias result from an inherited mutation. The rarity may be related to the observation that individuals with inherited cancers tend to die before reaching reproductive age (Segel and Lichtman, 2004). Of the leukemia-predisposing syndromes, Down syndrome is among the most common occurring in approximately 1 of 800 live births (Hill *et al.*, 2003). Children with Down syndrome are at an elevated risk of developing both ALL and AML with a cumulative risk of 2.1% by age five and 2.7% by age 30 (Hasle *et al.*, 2000; Hill *et al.*, 2003). Approximately 10% of children with Down syndrome are also born with a transient myeloproliferative disorder which resolves spontaneously within several months (Izraeli, 2006). Among

those that develop AML, most (approximately 1% in total) develop acute megakaryoblastic leukemia (AML M7), a rare and unique form of AML. Both the transient myeloproliferative disorder and the megakaryoblastic leukemia are characterized by a mutation in *GATA1*, a transcription factor that regulates megakaryocyte proliferation and differentiation (Izraeli, 2006). The ALL that develops in Down syndrome patients has a similar distribution that that seen in non-Down syndrome children with a median age distribution at 3-4 years (NCI, 2006).

Prenatal origins of ALL and infant AML

Over the past ten years, substantial evidence has accumulated indicating that for a number of leukemic subtypes, the initial disease-related genetic alterations occur in utero. The initial evidence came from studies of identical twins with leukemia (Greaves *et al.*, 2003; Greaves, 2005). Concordance rates for infants with ALL is very high (>50%) and exceeds the 10% seen in typical ALL and the <1% seen in adult ALL or AML (Greaves, 2005). Molecular and cytogenetic studies demonstrated that the leukemia-associated fusion genes occurring in most twins with concordant leukemia were identical. Since the fusion gene breakpoints are random within the affected region in other ALLs, the induced alterations indicated that both leukemias developed from a single clonal cell that originated in one twin in utero and whose clonal progeny had spread to the other twin via placental anastomoses (Greaves, 2005).

Evidence for a prenatal origin for most non-twin cases of childhood leukemia was provided by a retrospective analysis of archived neonatal blood spots (Guthrie cards) from a series of pediatric leukemia patients. The neonatal blood spots were evaluated using a polymerase chain reaction (PCR) technique for the presence the leukemia-specific clonal fusion gene sequences for a series of children who had developed leukemia at various ages (McHale and Smith, 2004; Greaves, 2005). In most cases, the fusion gene sequences unique to the leukemic clone were present within the blood spots collected at birth. A similar prenatal origin has been reported for leukemias characterized by hyperdiploidy as well as genomic instability (Kempski *et al.*, 2003; Maia *et al.*, 2004). The presence of functional leukemia-related fusion genes has also been seen in cord blood samples (Mori *et al.*, 2002). In the studied cases, the onset of leukemia has been

quite variable. In most, the leukemia developed with a relatively short latency (6 months for infant AML, and 2-5 years for childhood ALL). However in several cases, there was more than a ten-year span between birth and the onset of the leukemia (Greaves, 2005). These results demonstrate that most cases of childhood leukemia are prenatal in origin. However, additional cellular and genetic changes must occur for pre-leukemic clone to develop into the overt leukemia, and the necessary changes can occur rapidly or take many years to develop. Consistent with this two (or more) stage model, direct evidence for the presence of secondary postnatal genetic alterations has been seen in twin studies in which the paired samples share the identical initiating event but have distinctive secondary events, such as different TEL deletions or other chromosomal changes (Kempski *et al.*, 2003; Greaves, 2005).

The concordance data in twins with ALL implies that for every child with ALL there should be a significant number of healthy children who were born with a clinically silent preleukemic clone (Greaves *et al.*, 2003). To determine if preleukemic cells could be detected in healthy children, Greaves and co-workers screened a series of unselected cord bloods for the presence of *TEL/AML1* or *AML1-ETO* sequences using PCR techniques (Mori *et al.*, 2002; Greaves and Wiemels, 2003; Greaves, 2005). In confirmed positive cord bloods, they enriched, identified and counted cells with visible translocations using a combined fluorescence *in situ* hybridization (FISH)/immunophenotyping approach. These results showed that approximately 1% of newborns had a *TEL-AML1* positive B lineage clone and 0.2% exhibited an *AML1-ETO* positive clone. The observed frequency is ~100-times the incidence of *TEL-AML1* positive ALL (~1 in 12,000) or *AML1-ETO*-positive AML (~1 in 80,000), and indicates that leukemia initiation by chromosomal alterations is far more common than suggested by the overall rarity of the diseases (Mori *et al.*, 2002; Greaves and Wiemels, 2003; Greaves, 2005). Other fusion gene sequences have also been seen in the nucleated blood cells of adults although the functional and clinical significance of these low copy sequences is uncertain (Mori *et al.*, 2002).

These observations suggest a model for childhood leukemia in which the early genetic alterations or initiating events occur prenatally and are relatively common events (Greaves, 2005). After birth, additional genetic changes need to occur to convert or

allow the expansion of the covert preleukemic clone into a clinically observable leukemia. These secondary postnatal events are believed to occur less frequently. This model also implies that both *in utero* and postnatal exposures to environmental agents may play a role in the development of childhood leukemia.

Etiologic agents

In spite of the large number of studies have been performed to understand the origins of childhood leukemia, the specific causes of most leukemias remain unknown. A number of environmental, demographic and genetic risk factors (listed in Table 7 by degree of certainty) have been associated with increased risk. Although some environmental agents have been implicated as playing a role, the number for which there is strong evidence or that are widely accepted is quite limited. Three classes of etiologic agents – infectious diseases, ionizing radiation and chemotherapeutic agents - for which there is strong or increasing evidence of an association with childhood leukemia are briefly discussed below.

Infection hypotheses

Infectious agents have long been suspected as playing a role in the etiology of pediatric leukemias, particularly childhood ALL. Two major hypotheses have been advanced to explain how infections may contribute to childhood leukemias (McNally and Eden, 2004; Greaves, 2006): 1) Kinlen proposed that leukemia arises as a rare consequence of exposure to an as-yet-unrecognized, but relatively mild, infection and that an excess of childhood leukemia would be seen in locations that had an unusual type of population mixing; and, 2) As described above, Greaves proposed that precursor B-cell subtype of ALL occurs as a consequence of at least two independent mutations, one that occurs in utero and a second one that occurs later in childhood that precipitates the onset of the overt leukemia. Greaves hypothesized that common infections have a crucial role in promoting the critical second mutation and/or proliferation. According to his hypothesis, a delayed exposure to infection by an inadequately stimulated immune system could lead to an increase in the number of previously mutated preleukemic cells,

and increase the likelihood that the second critical mutation would occur (McNally and Eden, 2004; Ma *et al.*, 2005).

To date, no specific virus has been definitively linked with childhood leukemia and there is no evidence of viral genomic inclusions within the leukemic cells (McNally and Eden, 2004). Consequently, direct support is currently lacking for the Kinlen hypothesis. However, there is support for some aspects of this hypothesis such as the association with population mixing and perhaps a less direct role for infection (Bhatia and Robison, 2003; Kroll *et al.*, 2006). It is important to realize that the Greaves and Kinlen hypotheses are not mutually exclusive, and since elements of each may contribute to individual cases, it may be difficult in many cases to distinguish between the two. In recent years, substantial evidence has been generated in support of the Greaves hypothesis. This includes studies showing that the risk of childhood leukemias is inversely associated with early daycare attendance, a history of early common infections, more crowded households, higher rates of immunization, a history of breastfeeding, and urban-rural population mixing (Martin *et al.*, 2005; Greaves, 2006).

Ionizing Radiation

As a consequence of its prominent medical, military and energy-related uses, large numbers of children and adults have been exposed to ionizing radiation and its adverse effects have been extensively documented (BEIR V, 1990; UNSCEAR, 1994; Weiss *et al.*, 1995; Ron, 1998; Ron, 2003). Exposure to ionizing radiation has been shown to result in numerous types of cancer including several types of leukemia as well as other adverse effects that are likely to be involved in leukemogenesis such as hematopoietic toxicity, immune suppression and genetic toxicity leading chromosomal and gene mutations. The strongest association between radiation and cancer has been seen for leukemia and it has been consistently seen in numerous population studies including those exposed through medical, military and environmental exposures (BEIR V, 1990; UNSCEAR, 1994; Weiss *et al.*, 1995; Ron, 1998; Ron, 2003). The actual risk of these malignancies following exposure to radiation appears to be complex and related to the type of radiation, the dose, the proportion of the body exposed, and the extent of cell killing and DNA repair (Curtis *et al.*, 1994). Host factors such as age, sex, genetic

make-up, and physiological condition of the exposed individuals can also influence the risk of radiation-induced leukemia.

Children, in particular, have been shown to be at an elevated risk for radiation-induced leukemia as seen in the Life Span Study of the survivors of the atomic bombings in Hiroshima and Nagasaki. Among the survivors, who were exposed to both gamma, and to a lesser extent, neutron radiation, the highest leukemia risks were seen in children (Preston *et al.*, 1994; Preston *et al.*, 2004). Significant dose-related increases were seen for ALL, AML and CML, with the excess risks for ALL and CML being approximately 100% higher in the exposed males than in the females. When the leukemias were reclassified using the FAB classification scheme, all AML subtypes were present with a predominance of myeloblastic leukemias with and without maturation (M1 and M2) (Matsuo *et al.*, 1988). Among the ALL cases, most cases exhibited the L2 subtype but a significant portion also showed the L1 subtype. Latency periods were shorter for those under 15 years of age with a leukemia peak at 5-7 years post exposure as compared to later peaks for those exposed at later ages (Kamada and Tanaka, 1983). The leukemia risk also decreased more rapidly in the children as compared to those in older age groups. Overall, the risks to children were highest during the period from 1950 to 1965, and returned to near background levels thereafter (Preston *et al.*, 1994).

Similarly, the use of radiation for medical purposes has been associated with increases in childhood leukemia. Increased risk of leukemia has been reported following the use of radiation for diagnostic tests, for the treatment of benign diseases, and following radiotherapy for cancer (Ron, 1998; Ron, 2003). For hematopoietic and lymphoid tissues that are diffuse, a significant portion of the tissue must be irradiated to increase the incidence of neoplasia (Storer *et al.*, 1982). The leukemia risks from medical irradiation appear to have diminished in recent years due to a use of lower and more restricted doses as well as changes in therapeutic strategy in which high doses are applied within in limited fields. High radiation doses (above 3-4 Gray) result in extensive killing of marrow-containing progenitor cells, and have been associated with a reduced risk of developing leukemia (Boice *et al.*, 1987; BEIR V, 1990; Curtis *et al.*, 1994).

The leukemia risks associated with in utero exposure to ionizing radiation have been the subject of numerous investigations and reviews (Delongchamp *et al.*, 1997; Doll and Wakeford, 1997; Boice and Miller, 1999; Wakeford and Little, 2002). As described by Doll and Wakeford (Doll and Wakeford, 1997), the consistent association that has been seen in many case-control studies, suggests that, in the past, a radiographic examination of the abdomen of a pregnant woman, particularly in the last trimester, produced an approximately 40% increased risk of leukemia in the child. While generally accepted, this association continues to be controversial due to variety of concerns including recall bias, potential for confounding, and inconsistencies in the data (Doll and Wakeford, 1997; Boice and Miller, 1999). Another concern is that no increase in childhood leukemia was seen among the atomic bomb survivors. However, the expected number is quite small and within the confidence limits of other positive studies (Wakeford and Little, 2002). It should be noted that an increase of borderline significance ($p=0.054$) in the death rate from leukemia was seen in adult atomic bomb survivors who were exposed in utero (Delongchamp *et al.*, 1997). However, the increase consisted of only two cases, who died at ages 18 and 30, and who were exposed to relatively low radiation doses.

Chemotherapeutic agents

Following the initiation of intensive chemotherapy with genotoxic agents, an increase in therapy-related leukemia began to appear (Seiber and Adamson, 1975; Kantarjian and Keating, 1987; Levine and Bloomfield, 1992; Leone *et al.*, 1999). These leukemias, also known as treatment-related or secondary leukemias, consisted primarily of AML, although a small increase in therapy-related ALL has also been seen (Hunger *et al.*, 1992; Andersen *et al.*, 2001). With increased periods of follow-up, increases in other types of solid tumors have also been observed (Tucker *et al.*, 1988; Loescher *et al.*, 1989; Boffetta and Kaldor, 1994; van Leeuwen *et al.*, 1994). Interestingly, no increase in childhood leukemia has been reported in children who were exposed to chemotherapeutic agents *in utero* (Aviles and Neri, 2001; Cardonick and Iacobucci, 2004). In a few cases, infants whose mothers underwent chemotherapy late in pregnancy were born with mild to severe cytopenias, but no resulting leukemias have been reported (Garcia *et al.*, 1999;

Cardonick and Iacobucci, 2004).

Over time as new agents and therapeutic strategies have been employed, additional agents have been recognized as inducing leukemia in humans. Indeed, chemotherapeutic drugs comprise the largest group of agents generally recognized as human leukemia-inducing agents (Table 8). Currently, therapy-related leukemias constitute ~10% of the leukemia cases seen at major medical institutions (Pedersen-Bjergaard *et al.*, 1995; Leone *et al.*, 1999).

The identification of specific agents involved in leukemogenesis and the interpretation of many of the studies can be challenging due to the use of multiple therapeutic agents, varying dosing regimens, concurrent use of radiotherapy, and variable periods of patient follow-up. Over time, two main classes of leukemogenic chemotherapeutic drugs have been identified—alkylating agents and topoisomerase II inhibitors (Pedersen-Bjergaard and Philip, 1991; Pedersen-Bjergaard and Rowley, 1994). Chemotherapeutic drugs from these two classes are also leukemogenic in children, and will be briefly discussed in the following sections.

Alkylating agent-related leukemias

A large number of studies have demonstrated that patients treated with alkylating agent-based chemotherapy are at an increased risk of MDS and AML (Levine and Bloomfield, 1992; Pedersen-Bjergaard and Rowley, 1994; Smith *et al.*, 1994). These risks have been seen for both children and adults and are strongly related to the cumulative dose of the alkylating agent (Pedersen-Bjergaard and Philip, 1987; Tucker *et al.*, 1987; Hunger *et al.*, 1992; Pedersen-Bjergaard *et al.*, 2000; Pyatt *et al.*, 2005). The administered agents also exhibit varying degrees of hematopoietic toxicity, immunotoxicity and genotoxicity, which can also be affected by the agent, dose, and route of administration (Gale, 1988; Ferguson and Pearson, 1996; Sanderson and Shield, 1996). The incidence of treatment-related MDS and AML (t-MDS/t-AML) in adults has been reported to range from 1% to >20% (Felix, 1998). The incidences reported in childhood cancer patients have generally been lower ranging from approximately 0.6% to 4.2% in children treated for Hodgkin disease (Levine and Bloomfield, 1992; Barnard *et al.*, 2002). The 4.2% actuarial increase for Hodgkin disease corresponds to a relative risk

of 140. Whether these differences reflect fundamental differences in susceptibility or simply differences in reporting, treatment regimens, etc., is not clear. However in general, the increased risks of leukemia seen in children following treatment with alkylating agents do not appear to be greater than those seen for similarly treated adults, and in some cases may be less (Levine and Bloomfield, 1992; Pyatt *et al.*, 2005). There is also some evidence that the risk to young children may be less than that seen for older children (Pui *et al.*, 1990; Jenkinson *et al.*, 2004; Pyatt *et al.*, 2005), although this relationship has not been seen in other studies (Tucker *et al.*, 1987). The increases in this type of therapy-related leukemia generally appear 1 to 2 years after treatment and may remain elevated for 8 or more years following the completion of chemotherapy (Pedersen-Bjergaard and Philip, 1991).

In adults, leukemias induced by alkylating agents exhibit characteristics that allow them to be distinguished from de novo leukemias, and those induced by topoisomerase II inhibitors (Pedersen-Bjergaard and Rowley, 1994; Eastmond *et al.*, 2005). These leukemias are typically myeloblastic with or without maturation (FAB M1 and M2 subtypes), and are characterized by trilineage dysplasia and clonal unbalanced chromosome aberrations, most commonly involving loss of the entire chromosome or part of the long arms of chromosomes 5 and 7 (-7, 7q-, -5, 5q-). These t-AML have a modal latency of ~5 years, and the onset of the actual leukemia is often proceeded by myelodysplasia. These patterns have been identified largely from studies of adults, but there is supportive evidence that t-AML induced by alkylating agents in children also exhibits these characteristics (Rubin *et al.*, 1991; Barnard *et al.*, 2002). It should be noted that AML induced by ionizing radiation tends to exhibit similar features (MDS, clonal unbalanced chromosomal aberrations, etc.) although the range of the induced FAB subtypes tends to be broader (Matsuo *et al.*, 1988; Philip and Pedersen-Bjergaard, 1988; Gundestrup *et al.*, 2000).

Topoisomerase II inhibitor-related leukemias

In the late 1980s, a new type of therapy-related leukemia was recognized that exhibited unusual features that differed from those previously seen following treatment with alkylating agents and radiation (Pui *et al.*, 1989; Pui and Relling, 2000). The

affected patients had been treated with etoposide or teniposide, two of a newly developed epipodophyllotoxin class of chemotherapeutic agents (Pedersen-Bjergaard and Philip, 1991). In the ensuing years, these epipodophyllotoxins have become widely used, especially for treating childhood cancers, and a large number of studies have been published establishing an association between treatment with these drugs and the subsequent development of leukemia (Haupt *et al.*, 1993; Smith *et al.*, 1994; Pedersen-Bjergaard *et al.*, 1995; Pui and Relling, 2000).

The leukemia risk following epipodophyllotoxin-employing therapy was reported in to be very high in early studies, with cumulative incidences approaching 19% in some treatment groups (Pui *et al.*, 1989). Interestingly, in some cases the risk of a secondary cancer appeared to be more closely related to the treatment regimen than to total dose (Pui *et al.*, 1991). Patients receiving epipodophyllotoxins on a weekly or twice-weekly basis had much higher cumulative risks (12.4%) than those receiving the drugs on a biweekly schedule (1.6%). Similar results have been seen in more recent studies (Smith *et al.*, 1999) although others have seen a correlation between cumulative dose and epipodophyllotoxin-induced t-AML (Neglia *et al.*, 2001; Le Deley *et al.*, 2003). With implementation of newer treatment protocols, the risk of t-AML is lower than previously reported, and within the range of that seen with alkylating chemotherapeutic agents (Smith *et al.*, 1993). There is also evidence that the combined treatment of topoisomerase II inhibitors with alkylating agents (or cisplatin) confers a greater risk of t-AML than seen with either type of chemotherapeutic agent alone (Sandoval *et al.*, 1993; Smith *et al.*, 1994; Blatt, 1995). In most reported cases, the incidence of t-AML induced by these drugs is estimated to range from 2 to 12% (Felix, 1998).

The epipodophyllotoxins exhibit moderate myelosuppression, identifiable chromosomal damage and their leukemogenic effects by inhibiting topoisomerase II through a process that involves stabilization of the DNA-enzyme complex (Smith *et al.*, 1994; Ferguson and Pearson, 1996). Topoisomerase II is a nuclear enzyme involved in a wide variety of cellular functions, including DNA replication, sister chromatid separation, and recombination (Anderson and Berger, 1994). Leukemias induced following treatment with epipodophyllotoxin-type topoisomerase inhibitors appear from 10 months to 8 years following the initiation of chemotherapy, with a median latency of 2

to 3 years (Smith *et al.*, 1994). The induced AMLs are primarily of the monocytic or myelomonocytic subtypes (M4 and M5) and are rarely preceded by a myelodysplastic phase, a pattern that differs significantly from alkylating agent or radiation induced leukemias (Pedersen-Bjergaard and Rowley, 1994; Smith *et al.*, 1994).

Infrequently, ALL has been reported in patients following treatment with both alkylating agents and topoisomerase II inhibiting drugs (Hunger *et al.*, 1992) (Andersen *et al.*, 2001). Although t-ALLs occur infrequently, they seem to be more common in children under the age of 15 who have previously been treated with a topoisomerase II inhibitor (Andersen *et al.*, 2001).

One of the unique features of t-AML, and to a lesser extent t-ALL, induced by the epipodophyllotoxin-type of topoisomerase II inhibitors is the presence of clonal balanced translocations involving the *MLL* gene (also known as *ALL-1*, *HRX* and *HTRX-1*) located on the long arm of chromosome 11 (11q23) in the leukemic cells. Cytogenetic studies of patients with leukemias induced by these agents have shown that in over 50% of the cases, the leukemic clone involved a balanced translocation affecting the 11q23 region and another chromosomal partner, usually t(6;11), t(9;11), and t(11;19) (Pedersen-Bjergaard and Rowley, 1994; Smith *et al.*, 1994; Canaani *et al.*, 1995). In children previously treated with topoisomerase inhibitors, up to 90% of the secondary leukemias have an 11q23 alteration (Canaani *et al.*, 1995). As indicated previously, to date 87 rearrangements involving the *MLL* gene have been identified and 51 of the translocation partner genes have been characterized at the molecular level (Meyer *et al.*, 2006). The four most common *MLL* translocation partner genes (*AF4*, *AF9*, *ENL*, and *AF10*) encode nuclear proteins that are part of a protein network involved in histone H3K79 methylation (Meyer *et al.*, 2006) indicating an important role for this pathway in epipodophyllotoxin leukemogenesis.

Numerous lines of evidence indicate that topoisomerase II as well as DNA repair enzymes such as those involved in non-homologous end joining play an important role in the formation of the 11q23 translocations. These have been summarized in series of recent reviews (Greaves and Wiemels, 2003; Aplan, 2006; Felix *et al.*, 2006; Zhang and Rowley, 2006). The presence of topoisomerase II recognition sites has been found adjacent to the translocation breakpoints as have recombinase recognition sites, *Alu*

sequences, DNase hypersensitive sites and scaffold attachment regions, and suggest that multiple types of damage and repair probably contribute to the generation of the observed translocations (Felix *et al.*, 2006; Zhang and Rowley, 2006). While many different types of sequence have been seen at the breakpoints, the observed translocation patterns are consistent with a mechanism involving non-homologous end joining (Greaves and Wiemels, 2003; Zhang and Rowley, 2006). It should also be noted that the *MLL* gene has been shown to be prone to breakage during apoptosis, and it has been proposed that the observed translocations occur in hematopoietic cells rescued at an early, reversible stage during the apoptotic process (Stanulla *et al.*, 1997; Betti *et al.*, 2005; Vaughan *et al.*, 2005; Basecke *et al.*, 2006a). A role for apoptosis has also been proposed in the formation of other leukemia-related translocations (Eguchi-Ishimae *et al.*, 2001).

Interestingly, translocations involving 11q23 are seen in ~80% of infant ALL and 80% of the FAB M4/M5 subtypes of AML seen in infants and young children with no history of chemical or radiation exposure (de novo leukemias) (Felix *et al.*, 2006). In addition, the translocation breakpoints within the MLL gene in these patients have been reported to cluster within the same region where translocation breakpoints cluster in epipodophyllotoxin-induced t-AML (Cimino *et al.*, 1997). As a result, investigators have postulated that exposure to environmental or dietary topoisomerase II inhibitors *in utero* may be involved in the genesis of these infant leukemias. Two epidemiological studies and some mechanistic studies have provided some support for this hypothesis (Ross *et al.*, 1996; Ross, 1998; Abe, 1999; Strick *et al.*, 2000; Spector *et al.*, 2005). More recent analyses have reported some sequence and processing differences in the translocation breakpoints of the infant leukemias and the t-AML (Felix *et al.*, 2006).

Other leukemogenic agents

In recent years, evidence has accumulated that catalytic inhibitors of topoisomerase II, such as the anthracycline and bisdioxypiperazine derivatives, can also induce secondary leukemias (Xue *et al.*, 1992; Zhang *et al.*, 1993; Blatt, 1995; Andersen *et al.*, 1998; Le Deley *et al.*, 2003). The leukemias induced by these agents are similar to the epipodophyllotoxins in that they have short latency periods and are infrequently preceded by myelodysplasia. However, they typically exhibit different types of clonal

balanced rearrangements {e.g. t(8;12), t(15;17) and inv(16)} and different FAB subtypes (M2 and M3). These agents are more commonly used in adults and while there is less evidence, they are likely to be leukemogenic in children.

There are also a number of environmental and industrial agents listed by IARC as established or probable human carcinogens (Table 8). For most, the primary evidence for the observed increases in leukemia has come form studies of occupationally exposed workers, and again, while there is limited evidence, it is also likely that at high exposure levels these agents would be leukemogenic in children. One agent for which there is some evidence is benzene. A considerable number of teenagers were represented in the Aksoy series of Turkish shoemakers who developed hematotoxicity and, in some cases, leukemia following occupational exposure to benzene (Aksoy *et al.*, 1974; Aksoy, 1988). For example, 45 of the 217 individuals in one study were in the 13 to 20 age bracket, and 23 of 51 workers with hematological abnormalities were in the 12 to 20 age range (Aksoy, 1988). The responses in the adolescents were not noted as differing from that of the adults. Similarly, Nigerian youth exposed to high concentrations of benzene-containing gasoline exhibited serious hematopoietic toxicity with responses which were not identified as differing from the adults (Niazi and Fleming, 1989). Further, several ecologic epidemiological studies have provided suggestive evidence an association between leukemia incidence and proximity to gasoline stations, road traffic, or other potential sources of benzene exposure (Crosignani *et al.*, 2004; Steffen *et al.*, 2004; Knox, 2005). A summary of the general characteristics of a number of leukemia-inducing agents in humans is shown in Table 9. With the exception of the epipodophyllotoxins, most of the described characteristics have been seen primarily in adults. They have also been seen to a limited extent in affected children. In the case of radiation, where the latency periods differ for adults and children, the children's value has been used.

Genetic pathways in treatment-related MDS and AML

In a recent update, Pedersen-Bjergaard and colleagues proposed a series of models for t-MDS/t-AML that involves eight separate genetic pathways with different biological characteristics and origins (Pedersen-Bjergaard *et al.*, 1995; Pedersen-

Bjergaard *et al.*, 2002). An illustration of the various pathways originating from either spontaneous alterations or those induced by therapeutic agents is shown in Figure 1. More recently, additional interrelationships between new and previously identified genetic alterations, leukemogenic agents, and types of leukemia have been reported (Christiansen *et al.*, 2001; Harada *et al.*, 2003; Christiansen *et al.*, 2004; Zheng *et al.*, 2004; Klymenko *et al.*, 2005; Rege-Cambrin *et al.*, 2005; Wiemels *et al.*, 2005).

Introduction to biomarkers

In epidemiology, biological markers, commonly abbreviated for convenience as biomarkers, are any substance, structure or process that can be measured in the human body or its products that can predict or influence the incidence or outcome of disease (IARC, 1997b). Biomarkers can also be measured in animal studies for many purposes. In toxicological studies, biomarkers are commonly used to investigate the progression of a disease, to identify underlying mechanisms, show relationships between dose and response, and to allow comparisons to be made between species. The use of biomarkers in animals is also used to validate their relevance and use in humans. The integration of both animal and human studies can be particularly valuable in the area of risk assessment. However, reliable animal models for childhood leukemia have not yet been fully developed or established. As a result, this report will focus almost exclusively on biomarkers in humans. In recent years, a number of monographs have been published that discuss in detail many aspects of the use of biomarkers in molecular epidemiology and risk assessment (IPCS, 1993; IARC, 1997a; IPCS, 2001; IARC, 2004). These provide an insightful and systematic approach to the topic, and the reader is recommended to refer to them for additional background information. This report is intended to build upon these more general reports, and focus more specifically on biomarkers associated with leukemia in children and adults. The examples used will primarily be based upon the agents described in the previous sections and those listed in Table 8 that have been shown to induce leukemia in children, or that are likely to be capable of doing so.

As described by the Committee on Biological Markers of the National Research Council, the development of a disease resulting from environmental exposures occurs

through a series of stages beginning with exposure, progressing through an internal dose to a biologically active dose, which then exerts early biological effects. These subsequently lead to altered structure and function, and ultimately, to frank clinical disease (NRC, 1987; Wang *et al.*, 2001). A simplified flow chart showing the general classes of biomarkers as well as specific examples related to monitoring childhood leukemia is shown in Figure 2. Each step in the process can be modified by host-susceptibility factors which can affect the rate of progression from one stage to the next.

Biological markers are often classified into three general categories – biomarkers of exposure, effect, and susceptibility (NRC, 1987; Wang *et al.*, 2001). Biomarkers of exposure are often subdivided into biomarkers of internal dose and of biologically effective dose (Figure 3). A biomarker of internal dose refers to the measurement of the agent of interest, its metabolite or a derived product in a tissue or body fluid that can serve as a measure of current or past exposures. This can also include pharmacokinetic data including half-life, peak dose, or cumulative dose. The biologically effective dose is the amount of the bioactive agent interacting with critical targets at the subcellular, cellular or tissue level or with an established surrogate. Chemcial adducts with protein or DNA would be a common example of this type of biomarker. Both biomarkers of dose should provide quantitative or qualitative relationships with the exposure concentration.

Biological markers of effect can be separated into biomarkers of early effect and those associated with a subclinical presentation of the disease exhibiting altered structure or function. A biomarker of early effect is a measure of the presence and magnitude of a biological response to an environmental agent that occurs shortly after exposure. These effects may be non-specific such as the measure of mutational events in an endogenous or reporter gene such as HPRT, or may reflect an early alteration directly related to the progression of the disease such as the detection of a clone containing a leukemia-related fusion gene. Biomarkers of effect may also be measured in surrogate cells or tissues that reflect, or are believed to reflect, changes that are occurring in the target tissue. For example, changes in lymphocytes or blood counts are often used to estimate effects occurring in the bone marrow. As the biologically active dose reaches a sufficient intensity or duration, the affected tissue will exhibit an altered function which may be manifested as subclinical presentation of the disease. A sustained disruption in

hematopoiesis subsequent to chemical exposure would also represent an example of a subclinical or clinical effect related to an increased risk of developing leukemia.

Some biological markers indicate individual or population differences that can affect the response to the environmental agent independent of the exposure being studied. An intrinsic genetic characteristic or a preexisting condition the results in an increased biologically effective dose or a response in the target tissue can be a marker of susceptibility. These can include heritable germline susceptibility genes including rare genes such as ataxia telangiectasia mutated (*ATM*) or *TP53* or common polymorphisms in low risk genes such as *NQO1* and *MTHFR* that can modify a person's risk of developing leukemia.

General issues associated with biomonitoring in children

Numerous issues arise when measuring biological markers (biomonitoring) in children. Some of these are general issues posed by biomonitoring people of any age but some are unique to working with children. Two recent reviews have been published that have highlighted unique aspects involved in conducting biomarker studies in children, and provide a review of the general literature in this area (Neri *et al.*, 2006a; Neri *et al.*, 2006b). These reviews form the basis and primary source for the text and issues discussed below.

The biological samples needed for assessing exposure and biological effects can be obtained from many tissues using a range of non-invasive and invasive techniques. The choice of specimen and method of collection depends upon the type of assay to be performed, the age of the group to be studied, and the individual's health. Exfoliated cells can be readily obtained from the mouth or urine using non-invasive techniques and may be sufficient for some types of research such as genotyping or measuring the presence of micronuclei, a type of cytogenetic damage. However, these cells cannot be grown in culture and so their uses are limited. The collection of blood samples, typically obtained by venipuncture, is more invasive and, for larger than microliter quantities, may require skilled personnel such as a pediatric phlebotomist. However, peripheral blood lymphocytes can be readily cultured allowing a broader range of cytogenetic and mutational assays to be performed. Bone marrow cells would ideally be used for

biomarker studies of childhood leukemia. However, since invasive techniques are required for their collection, these will not often be available. Since blood originates from the bone marrow, it is frequently used as a surrogate tissue to investigate changes occurring in the bone marrow. However, changes occurring in the marrow may not be readily detected in peripheral blood samples.

From an epidemiological perspective, there are a number of advantages as well as some disadvantages, in working with children. One major advantage is the reduced impact of traditional confounding factors such as cigarette smoking, alcohol consumption, or occupational exposures. Children, at least until adolescence, usually do not drink or smoke, nor are they likely to be exposed occupationally. However, they may be exposed to environmental tobacco smoke (ETS) and may have higher exposures to environmental agents because physiologically their intake of air, water and food is higher per unit body weight (USEPA, 1997). They may also have a higher intake of dust and soil because of behavioral differences such as crawling and increased hand to mouth activities. Another advantage of studying children is the relative ease of following study subjects of school age.

There are also a number of limitations or disadvantages in conducting biomonitoring studies with children. One is that the low levels of genetic damage in children generally reduces the statistical power of the study and necessitates that larger sample sizes be used. Children are also more prone to infections, and have a higher incidence of infectious diseases than adults. In addition, in studies with teenagers, the possible role of hormonal fluctuations may need to be considered.

There are also a number of ethical issues that are unique to working with children. While most biomarker studies focusing on environmental exposures improve our knowledge of environmental risks to children in general, and help to define baseline levels of genetic alterations in children, these rarely provide an immediate benefit for the study participants. As a result, due to ethical considerations, field studies on children are generally limited to those that do not expose the child to unnecessary risks. Clinical biomarker studies of children face many of the same issues but may provide a more direct benefit to the participating child. In consideration of these issues, guidelines have been prepared by the Council of International Organizations of Medical Sciences in

collaboration with the World Health Organization. These guidelines state, "The risk of interventions that are not intended to be of direct benefit to the child-subject must be justified in relation to anticipated benefits to society (generalizable knowledge). In general, the risk from such interventions should be minimal-that is, no more likely and not greater than the risk attached to routine medical or psychological examination of such children. When an ethical review committee is persuaded that the object of the research is sufficiently important, slight increases above minimal risk may be permitted." (Neri *et al.*, 2006a).

The use of genetic markers in environmental studies of children also raises unique issues relating to consent. Consent for the use of samples and data from children must be provided by their parents or the legal guardians. However, it must also provide the right of the child to opt in or out of the study, especially when samples are archived for use in new studies, years after collection. Consent is therefore an ongoing process in which children recruited into studies at an early age (or before birth) are able to modify as they grow older. Other issues unique to children include topics such as incentives to participate, the management of study protocols, additional issues related to informed consent, and the communication of results. For a more detailed discussion of these points, the reader is recommended to see the extended discussion in (Neri *et al.*, 2006a) and the references provided therein.

Biomarkers of internal dose

The measurement of a chemical or its metabolite in a body fluid (or tissue) can serve as a biomarker of internal dose and can help measure current or past exposures. These internal dose biomarkers can include pharmacokinetic data which strengthens the relationship between exposure and response. It should also be recognized that the various types of exposure biomarker have a unique quantitative relationship to exposure and can exhibit a unique time course in its response (IPCS, 1993). As a result, the rate of absorption, distribution, and elimination as well as the sampling time and analytical sensitivity should be considered in the interpretation of the quantitative relationship between exposure and selected biomarker. For example, biomarkers for chemicals that are cleared rapidly, such as exhaled benzene or urinary benzene or metabolites, will be

present shortly after exposure but may be undetectable at later times. Other biomarkers, such as albumin or hemoglobin adducts of benzene metabolites, may represent only a small fraction of the total internal dose but, because they have a long half-life in the body (relative to exposure frequency), may accumulate to detectable levels with continued exposure (IPCS, 1993).

Numerous studies have been performed to measure the internal dose of leukemia-inducing agents. For chemotherapeutic agents, these are often used to optimize dosage and improve therapeutic efficacy. For other agents, these measurements have typically been performed as part of occupational studies of exposed workers, and are used as an estimate of exposure. In a few cases, environmental measures have been performed on children. Several examples of biomarkers that have been used to monitor internal dose are presented below.

Chemotherapy based on alkylating agents or topoisomerase II inhibitors has been used for more than twenty years for the treatment of cancer and other diseases. To increase the drug's efficacy and understand its disposition within the body, pharmacological studies are typically performed, and as a result, detailed information on absorption, distribution, metabolism, and excretion is often available. As an example, a description of the disposition of busulfan (also know as myleran), an alkylating agent used for treatment of CML, is presented below. The information has been largely abstracted from a common medical reference (McEvoy, 2006). Following oral administration of a 4 mg dose, busulfan has been shown to be rapidly absorbed with a peak plasma concentration of 68 ng/ml being seen at 0.9 hr after dosing. Within the plasma, approximately 30% of the drug binds irreversibly to plasma proteins. The remainder is rapidly distributed to other organs where it undergoes biotransformation. Busulfan is extensively metabolized resulting in more than identified 12 metabolites including methanesulfonic acid and 3-hydroxytetrahydrothiophene-1,1-dioxide. In the liver, busulfan is primarily metabolized through glutathione conjugation, both spontaneous and glutathione *S*-transferase-mediated. While extensively metabolized, busulfan and its metabolites are only slowly excreted with 30-60% of the dose appearing in the urine within 48 hours. Less than 2% of the administered busulfan is excreted unchanged in the urine, and negligible amounts are excreted in the feces (McEvoy, 2006).

While the above information has been generated primarily in adults, additional supplemental information related to children may be available. For example, the pharmacokinetic disposition of busulfan in children differs from that of adults with a lower mean bioavailability and larger inter-individual variation (Hassan *et al.*, 1996; McEvoy, 2006). Similarly, a larger apparent volume of distribution is seen in children than in adults (DrugDEX, 2006). In addition, the rate of excretion has been shown to vary in some groups of children; some children exhibit more rapid excretion (certain genetic disorders) and others slower excretion (lysosomal storage disorders). However, apart from the recognition that higher cumulative doses confer higher risks, it is not established how the observed pharmacological differences would affect a child's risk of t-AML.

In contrast to the leukemogenic classes of drugs, detailed pharmacokinetic information is seldom available for other types of leukemogens. Of the other classes of leukemogenic agents, benzene has probably been the most extensively studied. Major efforts to develop biomarkers have also been undertaken for other agents such as 1,3-butadiene and ethylene oxide. Detailed studies of benzene metabolism and physiologically based pharmacokinetic modeling have been performed (Bois *et al.*, 1996; Smith and Fanning, 1997; Brown *et al.*, 1998). In addition, numerous studies have been conducted to identify and validate biomarkers in humans. While methods have been developed to measure the levels of benzene and its metabolites in human blood, the most commonly used biomarker for estimating benzene exposure and internal dose is the measurement of benzene metabolites in the urine of exposed individuals. The quantitation of benzene's phenolic metabolites (phenol, hydroquinone and catechol) in the urine has long been used as a biomarker of benzene exposure. These metabolites are frequently present at variable levels in unexposed individuals, most likely due to dietary intake (McDonald *et al.*, 2001), and are considered less-than-ideal biomarkers. More recently, the benzene metabolites t,t-muconic acid and S-phenylmercapturic acid have been shown to be more reliable, particularly for monitoring benzene exposures at low exposure concentrations.

Almost all of the development and validation of these benzene biomarkers has been performed in benzene-exposed workers. However, in recent years, these markers

have been used at times to measure environmental benzene exposures in children. For example, t,t-muconic acid was recently used to measure benzene exposure in 144 Italian children, 92 from an urban environment and 52 from a rural environment (Amodio-Cocchieri *et al.*, 2001). Quantifiable levels of t,t-muconic acid were found in 63% of the urine samples analyzed. The levels in the urban children (99 µg/L) were approximately two times those found in the rural children (48 µg/L), and the higher levels were associated with proximity of the child's home to traffic. In a similar study, t,t-muconic acid and hydroquinone were measured over five days in 21 child-parent pairs in Rouen, France (Kouniali *et al.*, 2003). Detectable levels of t,t-muconic acid and hydroquinone were found in 85% and 100% of the samples, respectively. Mean values of t,t-muconic acid and hydroquinone expressed in mg/g creatinine were 1.6 and 1.8-fold higher in the children than the parents. However, significant correlations were not seen between the urinary metabolite levels and benzene levels measured at various locations in the children's and parents' environments. This suggests that exposure to benzene or its metabolites was occurring through other routes or sources such as uptake through the diet. As indicated above, the diet is a common source of exposure to phenolic compounds including those formed from benzene as well as a source of sorbic acid which can be metabolized to t,t-muconic acid.

As indicated in Table 8, tobacco smoke is widely recognized as a human leukemia-inducing agent. For measuring exposure to environmental tobacco smoke (ETS), various biomarkers have been employed. These include measurements of cotinine, nicotine, carbon monoxide and thiocyanate in blood as well as urinary levels of tobacco-specific nitrosamines and hydroxyproline (Benowitz, 1999). Of these, measurement of the nicotine metabolite, cotinine, is probably the most widely used, due to its relative specificity and sensitivity. It can also be measured for days after exposure so it is preferable to nicotine, which is present for a much shorter duration.

Since nicotine is quite specific for tobacco, the measurement of cotinine in the blood, saliva or urine can be used to track both direct and indirect exposure to this carcinogenic and leukemia-inducing agent (CDC, 2005). Cotinine is particularly valuable for identifying exposure to environmental tobacco smoke. Depending upon the cotinine level in the sample, individuals can be confidently separated into three groups,

smokers, nonsmokers exposed to environmental tobacco smoke (ETS), and nonsmokers without ETS exposure. As described by the CDC (CDC, 2005), "Nonsmokers exposed to typical levels of ETS have serum cotinine levels of less than 1 ng/mL, with heavy exposure to ETS producing levels in the 1-10 ng/mL range. Active smokers almost always have levels higher than 10 ng/mL and sometimes higher than 500 ng/mL.

Cotinine has been commonly used to assess exposure to tobacco smoke, particularly when other biomarkers or disease outcomes are being measured (Finette *et al.*, 1997; Weaver *et al.*, 1998; Benowitz, 1999; Qu *et al.*, 2003; Hecht *et al.*, 2006). Cotinine has also been useful in measuring the exposure of nonsmokers to ETS and exposure trends over time. In 1991, nearly 90% of the U.S. population had measurable levels of serum cotinine in their blood. Since that time, the CDC in its National Health and Nutrition Examination Survey has found a 75%, 68% and 69% decrease in the median cotinine levels in nonsmoking adults, children and adolescents, respectively (CDC, 2005). Of concern, was the observation that the levels of cotinine in children and teenagers (3-19 years old) were roughly double those of adults (age 20 and above).

Biomarkers of a biologically effective dose

The biologically effective dose is the amount of the bioactive agent interacting with critical targets within the target tissue, or a surrogate such as white blood cells. For most leukemia-inducing agents, chemical adducts with DNA or protein represent the most common example of this type of biomarker, and are used as a measure of an agent's ability to reach the target tissue in a bioactive and reactive form. The formation of sister chromatid exchanges, a common cytogenetic endpoint typically measured in peripheral blood lymphocytes, is also now generally considered to be more a biomarker of exposure than a biomarker of effect (IPCS, 1993). These biomarkers serve as an intermediate between exposure and early response, and are commonly used to estimate potential effects occurring at low doses, and determine the shape of the dose-response curve in the low dose region. Historically, many of the binding studies employed radiolabeled chemicals as adduct levels were below the detection limits of other types of analytical instrumentation being used at that time. This generally restricted the studies to measuring adducts and dose-response curves in animal models. However, in recent years with

technological advances in analytical techniques and instrumentation, it is increasingly possible to measure DNA and protein adducts in humans and identify the specific adduct being measured (Phillips *et al.*, 2000; de Kok *et al.*, 2002; Singh and Farmer, 2006).

DNA and protein adducts can be repaired or degraded so that information on the kinetics of their formation and clearance is necessary to accurately evaluate the relationship between exposure, adduct level and response. DNA adducts represent precursor lesions rather than specific mutagenic or genotoxic effects, as adducts may be converted into mutations or DNA strand breaks, but can also be efficiently repaired or remain unchanged in less critical non-coding sequences of DNA. For many leukemogenic agents such as the alkylating agents, the formation of a DNA adduct is an essential step in the carcinogenic process. However for others such as benzene, the relationship is less certain.

Numerous studies have been performed to measure DNA and protein adducts in individuals exposed to leukemogenic agents. These have typically involved measuring adducts in blood (or urine) rather than bone marrow due to the difficulties in obtaining bone marrow samples. While most studies have been conducted in adult patients or workers, a number have been performed using samples from children. Several examples of studies from adults and children are presented below.

For leukemia-inducing agents, the measurement of DNA adducts in humans has generally focused on two groups, patients undergoing chemotherapy and occupationally exposed workers (Farmer *et al.*, 1996). For example, in studies of patients administered platinum-based therapies, very good correlations were seen between the cumulative dose of the drug and the measured adduct levels (Poirier *et al.*, 1985; Reed *et al.*, 1986; Poirier and Weston, 1996). Similar results were seen for patients treated with procarbazine whereas for those treated with dacarbazine, a saturation in the formation of adducts was seen (Kyrtopoulos *et al.*, 1993; Farmer *et al.*, 1996). For some adducts, a linear correlation has been observed between cumulative dose and adduct level whereas no correlation was seen with others (Farmer *et al.*, 1996). The kinetics of adduct removal differed by agent with some adducts being removed or repaired fairly rapidly (within days) while others were detectable at significant levels for weeks after exposure (Poirier *et al.*, 1985; Reed *et al.*, 1986; van Delft *et al.*, 1993; Farmer *et al.*, 1996). It is

recognized that while DNA adducts can serve as valuable biomarkers, their relationship with dose can, at times, be complex and caution should be used in drawing conclusions about biological effects from the results (Sander *et al.*, 2005).

More recent efforts have focused on measuring DNA adducts and their persistence in important cancer-related genes in the white blood cells of patients treated with alkylating agents. Souliotis and associates measured the formation of monoadducts and interstrand cross-links in *TP53* and *N-RAS* in patients with multiple myeloma treated intravenously with the alkylating agent, melphalan (Souliotis *et al.*, 2003). Peak levels of monoadducts and crosslinks were seen at 2 hr and 8 hr, respectively, and seemed to correlate with the patient's response to chemotherapy. Similar results were seen in a follow-up study by the same investigators in which increased DNA damage and slower repair within the TP53 gene correlated with tumor reduction and progression-free survival (Dimopoulos *et al.*, 2005).

The detection of DNA adducts or other biomarkers related to a biologically effective dose is more challenging in individuals or children with environmental exposures. DNA adducts, SCEs and protein adducts present at birth or shortly thereafter in newborns have also been used to estimate in utero effects of maternal smoking or maternal exposure to ETS (Neri *et al.*, 2006b). In one of five studies, a significant increase in DNA adducts was seen in peripheral or cord blood cells of newborns born to mothers who smoked or were exposed to ETS during pregnancy (Neri *et al.*, 2006b). In the other four studies, no increase was seen. Similar results were seen for SCEs where a significant increase was seen in one of five studies. In contrast, increases in hemoglobin adducts or albumin adducts were seen in five of five studies of similarly exposed newborns. Significant increases in hemoglobin or albumin adducts were also seen in children living with one or more parents who smoked (Neri *et al.*, 2006b).

Protein adducts have also been widely used to measure exposure to occupational and environmental leukemia-inducing agents (Farmer *et al.*, 1996; Osterman-Golkar and Bond, 1996; Boogaard *et al.*, 1999; Albertini *et al.*, 2003b; Qu *et al.*, 2003; Rappaport *et al.*, 2005). Ethylene-oxide-derived and 1,3-butadiene-derived adducts with hemoglobin have been used to monitor exposure in occupationally exposed workers (Farmer *et al.*, 1996; Boogaard *et al.*, 1999; Albertini *et al.*, 2003b). Among those not occupationally

exposed, ethylene oxide-derived adducts have been shown to correlate strongly with smoking habits, urinary cotinine levels, and the number of cigarettes smoked (Bono *et al.*, 2002). Probably the most comprehensive use and validation of protein adducts as biomarkers has been performed by Rappaport and associates as part of a series of biomarker studies of benzene-exposed Chinese workers (Rappaport *et al.*, 2005). Adducts of the reactive benzene metabolites, benzene oxide and 1,4-benzoquinone, with cysteine residues on serum albumin were used to monitor exposure in 160 benzene-exposed workers and 101 local controls. Adduct levels were significantly higher in the exposed workers than in the controls. The relationship between benzene exposure and adduct formation was distinctly non-linear exhibiting a departure from linearity in adduct formation at benzene concentrations greater that approximately 1 ppm. The authors concluded that the biologically effective doses of benzene oxide and 1,4-benzoquinone would be proportionally greater in those exposed to low rather than high concentrations of benzene.

In a new approach to identify biomarkers of exposure (and possibly effect), the influence of benzene exposure on the gene expression was recently determined by comparing mRNA levels in the peripheral blood mononuclear cells obtained from another group of highly exposed Chinese benzene-exposed workers with those of non-exposed workers by a genome-wide microarray analysis (Forrest *et al.*, 2005; Smith *et al.*, 2005b). Twenty-nine known genes were identified that were deemed to be highly likely to be differentially expressed. A follow-up analysis performed using 508 cytokine probe sets identified 19 known cytokine genes that were expressed differently between the exposed workers and the controls. Confirmation of these results was performed for six of the identified genes by real-time PCR and of these cytokines CXCL16, ZNF331, JUN and PF4 were found to be the most significantly affected. These results would suggest that altered expression of these genes could potentially serve as new biomarkers of benzene exposure.

Biomarkers of early biological effects

Many different types of biomarkers for detecting early biological effects have been tested. These range from non-specific measures of mutations or chromosomal

damage in blood cells to the detection of leukemia-related fusion genes in bone marrow samples. An overview of the major types of early effect biomarkers and their significance will be presented, as well as examples of their use in monitoring leukemia inducing agents. The biomarkers will be divided into two sections – one that covers general non-specific effects and another more detailed one which focuses on biomarkers that are more specific for induced leukemias. As with the biomarkers of exposure, each of the early effect biomarkers has a different time course for its appearance and return to normal. Most of the examples are from studies have been conducted in adults but some examples will also be presented involving samples collected from children.

Early effect biomarkers indirectly related to leukemia

Structural and numerical chromosomal aberrations

Increased frequencies of chromosome aberrations are commonly seen in the peripheral blood lymphocytes of workers, patients and others following exposure to leukemia-inducing agents. Structural aberrations are the most common type detected although increases in numerical aberrations may also occur. These aberrations are generally non-clonal in nature and result from direct DNA damage or genomic instability originating in either the lymphocytes or their bone marrow precursor cells. Traditional approaches to detect chromosomal damage in lymphocytes have involved evaluating Giemsa-stained chromosomes in metaphase cells. In recent years, additional approaches such as fluorescence *in situ* hybridization (FISH) based assays or the micronucleus (MN) assay have become increasingly applied. The MN assay and certain FISH assays such as those using centromeric- or region-specific DNA probes allow cytogenetic evaluations to be performed on interphase cells. Other FISH assays such as those employing whole chromosome probes allow translocations and other more stable types of chromosomal exchanges to be more easily identified.

Chromosomal aberrations represent one of the earliest applied biomarkers of early effects, and the one for which a relationship to future cancer risk has been the most firmly established. While early studies identified a strong relationship between carcinogen exposure and an increase in chromosomal aberrations, the results of recently completed prospective studies have demonstrated a consistent association between increased

frequencies of chromosomal aberrations, primarily structural aberrations, and future cancer risk. Bonassi, Hagmar and others (Hagmar *et al.*, 1998; Liou *et al.*, 1999; Smerhovsky *et al.*, 2001; Norppa *et al.*, 2006) have shown that individuals exhibiting elevated levels of structural chromosomal aberrations in their peripheral blood lymphocytes are at significantly higher risk for developing cancer. While an early report suggested that this association might be particularly strong for lymphohematopoietic neoplasia (Bonassi *et al.*, 1995), subsequent reports have indicated that the increased risk is for cancers in general, and is not closely related to this particular type of cancer. Clonal structural and numerical aberrations are also commonly seen in human leukemias, and many lines of evidence indicate that these play an important role in leukemogenesis (Yunis, 1983; Solomon *et al.*, 1991). These observations indicate that the induction of chromosomal alterations by leukemia-inducing agents is likely to be an important biomarker relevant to leukemogenesis.

As indicated above, numerous studies have shown that individuals exposed to leukemogenic agents exhibit increased frequencies of chromosomal aberrations in their peripheral blood lymphocytes. Using conventional cytogenetic techniques, increases have been seen in patients exposed to chemotherapeutic drugs (Seiber and Adamson, 1975; Robison *et al.*, 1982; Lambert *et al.*, 1984; Reeves *et al.*, 1985; Mamuris *et al.*, 1989), workers exposed to industrial chemicals (Zhang *et al.*, 2002; Qu *et al.*, 2003), tobacco smokers (Obe *et al.*, 1982; Littlefield and Joiner, 1986), and those with environmental exposures (Liu *et al.*, 2002). The observed increases are affected by the dose administered and duration of exposure with the types and frequency influenced by the period of time between exposure and measurement. For example, elevated levels of chromosomal aberrations such as acentric fragments, micronuclei, dicentric chromosomes, inversions, and translocations have been seen shortly after exposure in the lymphocytes and bone marrow of individuals exposed to high levels of radiation (Tanaka *et al.*, 1983; Bender *et al.*, 1988; Lucas *et al.*, 1992). Both stable (translocations and inversions) and unstable alterations (acentric fragments, micronuclei, and dicentric chromosomes) decrease with time after exposure. However, a portion of the induced stable alterations may be detected 30 to 40 years after exposure, and can serve as a persistent biomarker of radiation exposure and early biological effects (Lucas *et al.*,

1992; Straume *et al.*, 1992). Similarly, increased frequencies of translocations have been measured in the cultured lymphocytes of patients years following treatment with chemotherapeutic agents (Haglund *et al.*, 1980; Lambert *et al.*, 1984).

Over the past 15 years, there have been extensive efforts to develop and validate new molecular cytogenetic biomarkers, to integrate them with other types of exposure and susceptibility markers, and compare the new biomarkers with earlier more established biomarkers. Major cytogenetic biomarker studies were undertaken in populations exposed to radiation, benzene, and 1,3-butadiene with mixed results. For example, FISH with whole chromosome probes as well as several other biomarkers was successfully used to evaluate genetic damage and reconstruct exposures in radiation-exposed individuals in samples obtained years after the Chernobyl disaster (Jones *et al.*, 2002).

In another series of studies, a variety of new and established biomarkers were used to assess cytogenetic damage in several groups of benzene-exposed workers. In a comprehensive series of studies lead by investigators at UC Berkeley and the National Cancer Institute (UCB/NCI), significant dose-related increases in both structural and numerical aberrations were seen in the exposed workers using a variety of new FISH techniques (Zhang *et al.*, 2002). For example, using centromeric DNA probes for a range of individual chromosomes, significant increases in hyperdiploid cells were seen for all of the chromosomes evaluated. Elevated levels of hypodiploidy as well as translocations were also seen. Certain chromosomes, particularly those involved in leukemia cells exhibiting clonal aneuploidy, deletions or translocations (e.g chromosome 5 and 7 for aneuploidy and 8 and 21 for translocations), were reported to be involved more frequently than other chromosomes (Smith *et al.*, 1998; Zhang *et al.*, 1998). However it should be noted that in similar validation studies sponsored by the Health Effects Institute in which similar FISH methods were applied to detect hyperdiploid and hypodiploid cells in benzene-exposed workers, few exposure-related alterations were seen (Eastmond *et al.*, 2001; Qu *et al.*, 2003). However when a more conventional cytogenetic approach was applied in the HEI study, modest but significant dose-related increases in structural chromosome aberrations were seen (Qu *et al.*, 2003).

In contrast to these benzene studies, no increase in cytogenetic alterations was seen using either conventional or FISH techniques in Czech workers exposed to 1,3-butadiene (Albertini *et al.*, 2003b). In this case, the inability to detect cytogenetic alterations in the butadiene-exposed workers is likely due to the modest exposure levels present in the participating workplaces. While the new molecular biomarkers present clear advantages in some areas, it should be remembered that many of these biomarkers are still being validated for use in human populations, and that studies with these new biomarkers face many of the same challenges as more traditional epidemiological studies.

Increasingly, the frequency of micronuclei in cultured peripheral blood lymphocytes is also being used to measure cytogenetic damage occurring in adults with occupational, medicinal and environmental exposures (Bonassi *et al.*, 2003; Bonassi *et al.*, 2005). This approach has also being increasingly used to monitor damage occurring in children (Neri *et al.*, 2003). Increased frequencies of micronuclei have been seen in children undergoing chemical and radiation treatment (Migliore *et al.*, 1991; Neri *et al.*, 2003), in children exposed to environmental tobacco smoke (Neri *et al.*, 2006b) as well as in children exposed to radiation from the Chernobyl catastrophe and other accidental radiation exposures (Fenech *et al.*, 1997; Zotti-Martelli *et al.*, 1999; Neri *et al.*, 2003). Similarly, an increase in structural chromosome aberrations has also been reported in a series of studies of Czech children and teenagers exposed to formaldehyde in their school classrooms (Neri *et al.*, 2006b). It should be noted that the frequency of micronuclei increases with age in both children and adults, and it is important that this be considered when evaluating the effects of chemical and radiation exposures (Bonassi *et al.*, 2001; Neri *et al.*, 2005).

In a recent study of the effects of maternal cigarette smoking, cytogenetic analyses were performed on chromosomes of fetal amniocytes obtained by amniocentesis from smoking and non-smoking mothers (de la Chica *et al.*, 2005). A significant increase in the frequency of structural chromosome aberrations and chromosomal instability was reported in amniocytes obtained from the tobacco-exposed fetuses. The 11q32 chromosome region, a region frequently involved in infant and childhood leukemias, was reported to be the region most affected by tobacco exposure. Frequent alterations involving 5q31 and 17q21, two other leukemia-associated regions, were seen in cells

obtained from both the smokers and, to a lesser extent, the nonsmokers. While the results are intriguing, caution is probably warranted in drawing conclusions from these results (Demarini and Preston, 2005). In a separate cytogenetic study, the frequency of translocations in cord blood lymphocytes was shown to be higher in newborns born to mothers who smoked than in newborns of nonsmoking mothers (Pluth *et al.*, 2000).

Somatic mutations in reporter genes

Mutations arising in reporter genes of somatic cells in humans have been used to assess background mutation rates, and as a biomarker for exposure to carcinogenic agents (Albertini and Hayes, 1997; Kubota *et al.*, 2000). Mutations have been investigated in hemoglobin and glycophorin-A (GPA) genes in red blood cells, and in HPRT, HLA and TCR genes in T-lymphocytes with varying success. Two of the most commonly used assays are GPA mutations in erythrocytes and HPRT mutations in T-lymphocytes. Each of these assays has unique features, baseline mutation rates, and target sizes. A brief overview of the use of these two assays based on information and text extracted from (Albertini and Hayes, 1997) is provided below. For additional details, please refer to the original article or other reviews by Albertini and others (Albertini *et al.*, 1990; Cole and Skopek, 1994; Kubota *et al.*, 2000).

The GPA gene is located on chromosome 4q, codes for a polymorphic glycoprotein located on the surface of red blood cells, and spans 44 kb which makes it a large target for induced mutations. It exists as two co-dominantly expressed alleles (M and N) that are present in approximately equal frequencies. The GPA assay measures the frequency of variant cells that have lost expression of the M form in heterozygous individuals (~ 50% of the population). The variant cells are detected by flow cytometry using fluorescent antibodies that can distinguish the M and N forms. Loss of the M allele typically through a point mutation (labeled 0/N) can be distinguished from more complex mutations involving gain of another N allele (N/N) and can provide insights into the mechanisms underlying the induced mutations.

The X-linked HPRT gene also spans 44 kb and encodes a transferase enzyme which is constitutively expressed but dispensable in mammalian cells. HPRT phosphoribosylates hypoxanthine and guanine for conversion to iosinic acid, and is

required to convert purine analogs such as 6-thioguanine to their cytotoxic forms. Cells with normal HPRT activity are susceptible to the toxicity of 6-thioguanine whereas mutants are resistant. This differential sensitivity provides a basis for selection, and allows rare mutant cells to be identified.

Both the GPA and HPRT assays have been used to detect mutations in individuals exposed to a variety of leukemia-inducing agents. Significant increases in mutant frequency have been seen in survivors of the atomic bomb, individuals with accidental and medical radiation exposures, patients administered chemotherapeutic drugs, and benzene-exposed industrial workers. In general, the responses increase with dose and return to baseline or near baseline levels after exposure has ceased. However, the relationship between dose, time since exposure, and mutation frequency can be complex. For example, substantial increases in GPA variant frequencies with high variability have been reported in atomic bomb survivors. Since these increases were measured forty years after the explosion, this indicates that the GPA biomarker can be long lived. The persistence of the marker and the high variability observed are consistent with radiation-induced mutations occurring in the relatively small number of bone marrow stem cells that survived exposure. In contrast, only a minor increase was seen when the HPRT assay was used to measure mutant frequencies in the atomic bomb survivors. Initially this was thought to be due to an insensitivity of HPRT assay to radiation-induced mutations. However, a more recent interpretation attributes this to a loss of mutant T-lymphocytes in the 40 years between exposure and testing. In survivors who were young at the time of exposure, the HPRT mutations are believed to have occurred in the committed and multipotent bone marrow precursor cells, and this is the reason for the small elevation that was seen. However in contrast to the atomic bomb survivors, patients receiving localized radiation for cancer treatment have not shown an increase in GPA variant frequencies. In this case, the lack of an increase is likely due to either a lack of bone marrow cells within the targeted field, or extensive killing of the bone marrow precursor cells that were present (Grant and Bigbee, 1994; Mott *et al.*, 1994).

As indicated above, significant increases in GPA and HPRT mutant frequencies have been seen in both children and adult patients treated with chemotherapy. However, while both children and adults seem to respond similarly to exposure, there are significant

differences in the persistence of the variant cells in the treated adults and children (Albertini and Hayes, 1997). Within several months of treatment and consistent with the erythrocyte lifespan, the levels of GPA variants in adults return to baseline levels. In contrast, elevated levels of GPA variants have been seen for over 10 years in children following chemotherapy (Hewitt and Mott, 1992; Boyse *et al.*, 1996). Interestingly, three of the patients in the Boyse study developed t-AML, all of whom exhibited elevated variant frequencies at the diagnosis of AML. Greater persistence in the HPRT mutagenic response has also been seen in children following treatment with chemotherapeutic agents. The results of both the GPA and HPRT assays suggest that in children as compared to adults, a relatively larger proportion of the induced mutations may occur in the bone marrow stem cells (Albertini and Hayes, 1997).

The HPRT assay has been used in several studies to evaluate the effects of maternal cigarette smoking on the somatic mutant frequencies in healthy newborns (Neri *et al.*, 2006b). In an initial report using an autographic method, an elevation in HPRT variants was seen in the cord blood lymphocytes obtained from infants born to smoking mothers as compared to those born to non-smokers (Ammenheuser *et al.*, 1994). An increase was also seen in the smoking mothers and the frequencies correlated with the number of cigarettes smoked. In a similar biomarker study by Manchester and colleagues, an increase was seen when comparing the HPRT mutant frequencies of newborns whose mothers smoked with those whose mothers were not exposed to ETS (Manchester *et al.*, 1995; Neri *et al.*, 2006b). However, no increase was seen when those exposed to smoke were compared to a combined group comprised of those exposed to ETS and the nonsmokers. No increase in variant frequency was also seen in the GPA assay.

In a similar study using the HPRT cloning assay, a significant increase in mutant frequency was not seen in the cord blood lymphocytes of newborns born to mothers who smoked during pregnancy or who were exposed to environmental cigarette smoke (Finette *et al.*, 1997). However, in a detailed follow-up study of the individual HPRT mutants, a significant difference in the mutational spectrum was seen in the lymphocytes of newborns exposed to cigarette smoke in utero (Finette *et al.*, 1998). The most notable difference was an increase in “illegitimate” deletions mediated by V(D)J recombinase in

the in utero-exposed newborns. The authors noted that deletions mediated by V(D)J recombinase are the most characteristic mutations observed in newborns and children and have an age-related incidence. The age-specific distribution of illegitimate V(D)J recombination correlates with the age distribution of childhood ALL, and when occurring in cancer-related genes, is believed to play an important role in leukemogenesis (Finette *et al.*, 1998).

Leukemia-related biomarkers

Myelosuppression and immunotoxicity

Myelosuppression and immunotoxicity frequently accompany exposure to leukemia-inducing agents, particularly those such as the chemotherapeutic drugs, ionizing radiation and benzene for which leukemogenicity has been clearly established (Eastmond, 1997). The number of individuals affected and the magnitude of toxicity is influenced by the agent and dose-related factors such as total dose, dose per treatment, schedule and route of administration as well as individual host factors (genetic susceptibility, prior therapy, health status, etc.) (Gale, 1988). A brief overview of the myelosuppressive effects of cancer therapeutic drugs has been written by Gale (Gale, 1988), and is the basis for the following description. The severity of myelotoxicity induced by antineoplastic drugs varies considerably by chemical class. For drugs associated with t-AML, moderate to severe effects are generally seen. The epipodophyllotoxins, cisplatin and procarbazine typically produce more moderate toxicity whereas severe effects are more common for the alkylating agents, the anthracyclines, and the nitrosoureas.

The period between dosing and the onset or appearance of the myelosuppression is also related to the class of agent. For some agents such as ionizing radiation, the onset of myelotoxicity occurs within 0 to 48 hr after exposure. For others, longer periods are required. The onset of myelosuppression by the alkylating agents and anthracyclines occurs 1 to 3 weeks following exposure, and is believed to be due to the effect of these agents on immature hematopoietic cells that becomes more evident as the more mature blood cells die and require replacement. Myelosuppression induced by the nitrosoureas and mitomycin C is less frequent and occurs 4 to 8 weeks after treatment. This delayed

effect is believed to be due to a relatively selective effect on immature stem cells. However, the onset of this delayed effect is dose-dependent. A two to three-fold increase in dose reduces the onset of myelotoxicity to one week. For some drugs such as busulfan, the manifestation of the myelotoxic effects is considered to be latent and may only be manifested only under stress-related conditions.

In most cases, the induced myelotoxicity is transient with the blood cell counts returning to normal following the cessation of treatment or exposure (Hendry and Feng-Tong, 1995; Irons *et al.*, 2005). However in some cases, the induced myelosuppression can be more persistent and progress to pancytopenia or infrequently to aplastic anemia, a condition that confers a much greater risk of developing leukemia (~10%) (Aksoy *et al.*, 1984; Jandl, 1987; Ohara *et al.*, 1997; Imashuku *et al.*, 2003). Increased risks have also been seen for those who have previously exhibited less severe forms of bone marrow toxicity. Studies of benzene-exposed workers have shown that the leukemia mortality rate was much higher for workers who had previously been diagnosed with bone marrow poisoning (700 per 10^6 person-years) as compared to those exposed to, but not poisoned by, benzene (14 per 10^6 person-years), and particularly as compared to the general population (2 per 10^6 person-years) (Yin and Li, 1994). In one report, 36% of the benzene leukemia cases had a history of benzene poisoning with leukopenia or pancytopenia (Yin *et al.*, 1994). However, this also indicates that for most cases, clinically detectable myelotoxicity may not be observed. It should also be noted that at lower exposure levels, the decreases in cell counts occurring in exposed groups may fall within what is considered the normal clinical the range (Qu *et al.*, 2002; Lan *et al.*, 2004). This highlights one of the challenges in using myelotoxicity as a biomarker, as the normal range varies considerably in adults. For example, the mean white blood cell count in adults is 7,200 x 10^3/µl with a 95% range from 3,900 to 10,900 x 10^3/µl (Jandl, 1996). This may be even more problematic for infants and children as the normal range of blood cells counts varies as well and decreases with age (Jandl, 1996).

Leukemia-related genetic biomarkers

As outlined above, a large number of clonal cytogenetic alterations and mutations have been seen upon clinical presentation of the leukemia in both children and adults.

These genetic changes often play critical roles in the development of leukemia, and numerous attempts have been made, with varying success, to detect and monitor these alterations and mutations at earlier stages in the development of leukemia. Because their involvement in leukemogenesis, the detection of these biomarkers should provide valuable insights into the early development of the disease, and more allow individuals at elevated risk to be more accurately identified. Two general approaches have been employed. In the first, genetic studies have been performed on individuals with current or past exposure to a known leukemia-inducing agent and the presence of unusual, non-random, or leukemia-related changes has been noted. In the second approach, investigators have looked specifically for leukemia-related genetic changes in individuals at elevated risk or in archived samples of those who subsequently developed the disease. Examples of both types of approaches are presented below.

In cytogenetic studies of the peripheral blood lymphocytes of highly exposed atomic bomb survivors conducted thirty plus years after the explosion, a non-random distribution of breakpoints was reported (Tanaka *et al.*, 1983; Kamada *et al.*, 1987). A number of these nonrandom breakpoints (e.g., 5q31, 7q32, 8q22, 8q24, 11q23, 21q2, 9q34) lie within or immediately adjacent to regions that are frequently affected in therapy-related and de novo leukemias and may represent early cancer-related changes. Elevated frequencies of alterations affecting 5q31, 7q22, and 21q22 also have been seen in the lymphocytes of ankylosing spondylitis patients many years after radiotherapy (Buckton, 1983) and translocations involving 7q32-36 have been observed in the lymphocytes of technicians with long-term radiation exposure (Kumagai *et al.*, 1991).

Similar studies have been conducted in benzene-exposed workers and in workers who had been previously poisoned by benzene. No obvious breakage pattern has been seen, although a nonrandom distribution of chromosome breakpoints has been reported by some investigators (Sasiadek *et al.*, 1989; Li and Ding, 1990; Sasiadek and Jagielski, 1990; Sasiadek, 1992). In the studies by Sasiadek and associates, an excess breakage was reported for chromosomes 2, 4, 7 and 9. In a limited Chinese study, the lymphocytes of four patients and the bone marrow of one patient with chronic benzene poisoning exhibited deletions and gaps in the long arm of chromosome 6, a region frequently involved in lymphoid neoplasia (Li and Ding, 1990).

It should be noted that nonrandom breakage involving a number of the chromosomal bands indicated above are periodically seen in controls or individuals with no known exposure to leukemia-inducing agents. Many of these bands are considered fragile sites and their relationship to cancer has been the focus of many hypotheses and studies (Yunis and Soreng, 1984; Marlhens *et al.*, 1986; Richards, 2001).

Targeted studies of high-risk patients

In the second approach, investigators have specifically looked to determine if a disease-specific genetic biomarker was present, typically in peripheral blood or cord blood lymphocytes, prior to the onset of leukemia in high-risk individuals, or in archived blood and bone marrow samples from individuals who have developed leukemia. A prime example is the measurement of leukemia-specific fusion genes in blood spots archived at birth in children who subsequently developed leukemia. In a series of studies, Greaves and colleagues identified unique fusion genes formed by translocations in the leukemic cells of childhood leukemia patients (Greaves *et al.*, 2003; Greaves and Wiemels, 2003; Greaves, 2005). Using PCR primers specific for the patients' leukemic clones, the presence of the identical fusion genes was detected in neonatal blood spots from the patients that had been archived at birth, months to years prior to the clinical onset of leukemia. Similar results were seen using the detection of clonotypic IGH or TCR rearrangements to detect the presence of the preleukemic cell, including cases with hyperdiploid karyotypes (Yagi *et al.*, 2000; Taub *et al.*, 2002). Because the archived blood spots only contain ~30,000 nucleated cells and degradation of the DNA can occur, a negative test result cannot be interpreted with confidence (Greaves, 2005). However, the results to date indicate that the leukemic lesion is present in ~100% of infant ALL with the MLL-AF4 lesion, ~75% of cases of childhood ALL with the TEL-AML1 lesion, ~50% of childhood AML cases with the AML1-ETO lesion, and a majority of childhood ALL exhibiting hyperdiploidy (Taub *et al.*, 2002; Greaves and Wiemels, 2003; Greaves, 2005). A prenatal origin has also been shown for pediatric leukemias exhibiting translocations involving the PML-RARA and the CBFB-MYHII fusion genes (McHale *et al.*, 2003). In contrast, these studies have also shown that a similar fusion gene is infrequently seen in childhood ALL exhibiting the E2A-PBX1 fusion gene. These results

demonstrate that when a leukemic cell has a unique and known genetic rearrangement, it is possible to detect the presence of the sequences years before the clinical onset of leukemia.

Genetic biomarkers have also been used to monitor several groups of patients at high risk for developing t-AML resulting from intensive chemotherapy. Several studies have been performed to monitor translocations involving MLL fusion products following treatment with topoisomerase II inhibitors. A retrospective analysis was performed using cryopreserved and slide-affixed bone marrow cells obtained from a 13-year old AML patient who had previously been treated with topoisomerase II inhibitors (as well as other chemotherapeutic agents) for neuroblastoma (Megonigal *et al.*, 2000). Using a panhandle PCR approach and Southern blotting, the MLL-GAS7 fusion gene was not detectable in the bone marrow at the diagnosis of neuroblastoma, but was present 3, 7, 10, 11 and 12 months after diagnosis. An analysis of bone marrow aspirates on glass slides revealed that the lesion was present 1.5 month after the start of treatment. Interestingly, at this point the patient had only been treated with two courses of cyclophosphamide, doxorubicin, and vincristine. Monocytosis and cytopenias appeared at 4.5 months and AML (FAB M4) 15.5 months after the translocation was detectable by PCR.

In a similar study, PCR with patient-specific primers was used to detect MLL-ENL fusion genes in the bone marrow and blood samples previously obtained from a 16-year old boy with ALL who subsequently developed t-AML (Blanco *et al.*, 2001). The leukemic gene was not present in the bone marrow at the time of ALL diagnosis or 56 days after the start of chemotherapy. The MLL-ENL fusion gene was detected in the bone marrow after 6 and 20 months from the beginning of chemotherapy and at the diagnosis of t-AML (FAB M4). Of note,the fusion gene was not detected in blood samples at the time of ALL diagnosis or after 0.7, 2, 8, 10, and 12 months of therapy but was detected beginning 16 months after therapy and at t-AML diagnosis (23 months).

In a prospective study, Ng and associates monitored MLL cleavage and rearrangement using Southern blot analysis and panhandle PCR in serial samples from 71 children undergoing chemotherapy (Ng *et al.*, 2005). MLL cleavage was observed in six bone marrow samples from five patients beginning 2-10 months after the start of therapy. Three of the children with low levels of cleavage (23-30%) remained well and two were

receiving treatment at the end of the study. One child with two positive samples and a higher level of cleavage (45-48%) died from treatment-related toxicity and relapsed leukemia. The patient with the highest level of MLL cleavage (50%) at 3 months developed a t-AML with an MLL rearrangement 6 months after the start of treatment. In this case, the MLL rearrangement was only detected in the bone marrow at the onset of the t-AML. Only the germline MLL breakpoint-cluster-region fragment was seen at the initial evaluation in the five patients, and in all of the blood samples obtained for the entire cohort. There was no obvious relationship between between the degree of MLL cleavage and the cumulative dose or schedule of the topoisomerase II inhibitors administered.

Cancer patients receiving an autologous bone marrow transplant are at high risk of developing t-AML (up to 20%) and have been the focus of several biomonitoring studies. This technique is typically used for patients that have failed standard induction and salvage therapies. Consequently at the time of transplantation, the patients have been exposed to numerous treatments prior to the high dose chemotherapy used in the procedure. Morphologic and genetic markers have been monitored in bone marrow cells collected at the time of transplantation and at various follow-up times. Several of these studies, briefly described below, illustrate some of the promise and challenges associated with trying to monitor disease-related biomarkers in high risk individuals.

Amigo and associates performed a retrospective FISH analysis using the cryopreserved peripheral blood stem cells that were used for transplantation and showed that the clonal cytogenetic anomalies (-15 and dic(7;15) present at the diagnosis of t-AML were present in the stem cells at the time of transplantation, twenty-seven months earlier (Amigo *et al.*, 1998). The same alterations were also seen in bone marrow samples harvested 6 months after transplantation. Abruzzese and colleagues performed a retrospective analysis of morphologically normal pre-transplant marrow or stem cell specimens obtained from 12 patients who subsequently developed t-MDS (Abruzzese *et al.*, 1999). In 9 of the 12 cases, the same cytogenetic abnormality observed at the diagnosis of MDS was detected by FISH in the pre-high dose chemotherapy specimens. Similar results were seen by Mach-Pascual and co-workers (Mach-Pascual *et al.*, 1998). They performed a number of clonality assays on 78 patients following autologous bone

marrow transplantation. Of the 78 patients, 37 had cytogenetic analyses performed after the transplant, and 16 of these had a cytogenetically abnormal clone.

Bhatia and colleagues conducted a prospective study of morphological, cellular and genetic changes occurring in 103 patients undergoing autologous hematopoietic cell transplantation for lymphoma (Bhatia *et al.*, 2005). Eight patients eventually developed t-MDS/t-AML. These patients tended to have a reduced recovery of committed progenitor cells and a poor telomere recovery. Cytogenetically, in most cases, only cells with a normal karyotype were detected prior to the high dose chemotherapy performed shortly before transplantation, and at sampling times prior to the development of t-MDS/t-AML (for samples that were evaluated). In one case, the patient had an abnormal clone at the time of transplantation but exhibited a normal karyotype at the diagnosis of t-MDS.

In other cases, chromosomal instability has been observed so that clonal aberrations observed at an early stage in high-risk patients may not be detected at a later stage. For example, as part of a prospective study of 229 women treated with high dose therapy and autologous stem cell transplantation, cytogenetic analyses were performed on 60 women over a period of 12-59 months after the transplantation. None of the women exhibited a clonal leukemia-related aberration. However, three patients did show other types of clonal chromosomal abnormalities {two with trisomy X and one with a t(1;6)} and two exhibited non-clonal reciprocal translocations. Two of the patients with clonal aberrations had blood cytopenias as well as dysplastic features in the bone marrow. However, these were not classifiable as MDS. Similar dysplastic features were seen in four patients with normal karyotypes. All of the cytogenetic aberrations, with the exception of a +X detected by FISH in a residual cell population, were transient and disappeared. None of the 229 women developed t-MDS/t-AML within the period evaluated in the study.

In contrast, more striking results were seen by Lillington and associates in their studies of 230 patients who underwent high dose therapy followed by autologous hematopoietic progenitor cell support (Lillington *et al.*, 2001; Lillington *et al.*, 2002). Overall, thirty-three of the patients developed t-MDS/t-AML and 20 of these were screened for the presence of therapy-related abnormalities prior to the high dose therapy.

Another 24 high dose therapy patients without t-MDS/t-AML were used as controls. Using an interphase FISH assay with probes to detect the types of aberrations seen in t-AML following treatment with alkylating agents, significant levels of abnormal bone marrow cells were seen prior to high dose therapy and at intermediate follow-up times in 20/20 patients who developed the secondary leukemia as compared to 3/24 patients in the control group. The median time from high dose treatment to the development of t-MDS/t-AML was 4.5 years. Traweek et al. reported a 9% risk of developing clonal cytogenetic abnormalities typical of t-MDS at 3 years following high dose therapy, with ~50% of the patients with an abnormal clone developing t-MDS (Traweek *et al.*, 1994; Mach-Pascual *et al.*, 1998).

Targeted studies of exposed workers

As indicated previously, researchers have used interphase and metaphase FISH to investigate whether benzene-exposed workers exhibited altered levels of chromosomes implicated in secondary leukemias induced by benzene and other agents (Zhang *et al.*, 2002). In the UCB/NCI benzene studies, highly exposed workers exhibited significantly elevated frequencies of trisomy or hyperdiploidy for chromosomes 1, 5, 7, 8, 9, and 21 as well as monosomy for chromosomes 5, 7, and 8, but not for chromosomes 1 and 21 (Zhang *et al.*, 2002). Deletion of portions of the long arms of chromosomes 5 (5q31) and 7 (7q36-qter) as well as translocations between chromosome 8 and 21 were also reported to occur at increased frequencies in the exposed workers (Smith *et al.*, 1998; Zhang *et al.*, 1998). The elevation of these alterations in the workers combined with the recognition that a number of the observed alterations such as -5, -7, 5q-, 7q-, +8, t(8;21) are seen in treatment-related or de novo leukemias suggests that the observed alterations might contribute to benzene leukemogenesis and indicate individuals at a higher risk of developing leukemia. However, as mentioned previously, similar results for some of these FISH biomarkers were not seen in similar studies of benzene-exposed workers conducted by a different group of investigators (Qu *et al.*, 2003). Additionally it should be noted that the frequencies of hyperdiploid and hypodiploid cells in these studies are very high in both the exposed and the control workers and much higher than those seen in studies using conventional cytogenetic approaches. Secondly, while increased

translocations between chromosome 8 and 21 were seen in the exposed workers, only two cells observed in one individual involved the specific chromosomal regions involved in leukemogenesis. However, the presence of the fusion gene in this individual was confirmed by RT-PCR.

Genetic alterations in normal individuals

Ideally the levels of a disease-related biomarker should correlate well with the risk of developing the disease. Because of the rarity of leukemia, one might expect that the frequencies of leukemia-related biomarkers would be quite low in the general population. Alternatively, the biomarkers could be more common but additional uncommon genetic changes would need to occur for the altered cell to progress to a leukemic cell. This second scenario appears to be more likely as relatively high levels of many of these disease-related biomarkers have been detected in normal non-exposed individuals.

Greaves and colleagues investigated the frequencies of gene fusion products associated with childhood leukemia in a number of studies (Mori *et al.*, 2002; Greaves *et al.*, 2003; Greaves and Wiemels, 2003; Greaves, 2005). As indicated previously, these investigators screened approximately 600 unselected cord bloods for the presence of TEL/AML1 or AML1-ETO sequences using PCR techniques. In the confirmed positive cord bloods, they enriched, identified and counted cells with the associated translocations {t(12;21) and t(8;21)}using a combined fluorescence in situ hybridization (FISH)/immunophenotyping approach. The results demonstrated that approximately 1% of newborns had a TEL-AML1 positive B lineage clone and 0.2% exhibited an AML1-ETO positive clone. The observed frequency is ~100-times the incidence of TEL-AML1 positive ALL (~1 in 12,000) or AML1-ETO-positive AML (~1 in 80,000), and indicates that gene fusion by chromosomal rearrangement is far more common than suggested by the overall rarity of the diseases (Mori *et al.*, 2002; Greaves and Wiemels, 2003; Greaves, 2005). Other translocations including t(9;22), t(6;11), t(15;17) and the del(1p) as well as higher levels of t(12;21) have been reported in the literature (Eguchi-Ishimae *et al.*, 2001; Janz *et al.*, 2003; Smith *et al.*, 2005a). In most cases, these were detected in small numbers of individuals (Smith *et al.*, 2005a), or the transcripts were detected in a large

proportion of those tested but were present at much lower copy numbers (Basecke *et al.*, 2002). In some cases when much higher frequencies of the fusion genes have been reported in the general population (Uckun *et al.*, 1998), others have not been able to reproduce the results (Greaves *et al.*, 2003).

Other types of genetic alterations seen in leukemic cells have also been detected at high frequencies in normal individuals. For example, partial tandem duplications of the MLL gene detected by RT-PCR have been reported to occur at high, widely varying, frequencies (16-93%) in the blood and bone marrow of healthy individuals (Marcucci *et al.*, 1998; Schnittger *et al.*, 1998) and in the cord blood of infants (Basecke *et al.*, 2006b). However, differences have been seen between rearrangements seen in leukemic patients and those detected in healthy individuals, suggesting that the detected PCR products may not function in the same manner and/or may be splicing artifacts (Marcucci *et al.*, 1998). In addition, the levels of the MLL partial tandem duplication that have been reported as commonly detected are present at much lower levels in healthy individuals (Basecke *et al.*, 2006b).

In many cases, the presence of the fusion genes in normal individuals has been based on the detection of the fusion gene transcripts by nested RT-PCR and has not often been followed by sequencing of the cDNA (Janz *et al.*, 2003). Furthermore, confirmation by obtaining the reciprocal fusion transcripts or the corresponding genomic sequences has not been obtained. As a result, the quality of the evidence in many cases is considered to be quite weak (Janz *et al.*, 2003). The retrospective studies by Greaves and associates are a notable exception, and considered to be of high quality. Also, a number of these quality issues seem to have been addressed in more recent studies (Basecke *et al.*, 2006b).

Similarly, the frequencies of hyperdiploidy and hypodiploidy detected using FISH in both the exposed and control subjects are quite high in the reported studies, and most likely reflect other phenomena in addition to the actual chromosome numbers (Eastmond *et al.*, 1995). When using FISH, problems can occur in measuring both chromosome loss and gain and the results can be significantly influenced by numerous technical factors (e.g. probe quality, harvest and hybridization conditions, DNA staining, overlap of signals, scoring criteria, etc.). It should also be noted that technical problems such as

artefactual chromosome loss or inadequate cell proliferation can also affect conventional cytogenetic analyses. Sample size can also be an issue. Typically, only 20 metaphase cells are evaluated clinically, so aberrant cells with low levels of aberrations or cells that replicate slowly in culture may not be detected. This may have a significant effect on the interpretation of the usefulness or feasibility of using a biomarker. For example, in the Bhatia study (Bhatia *et al.*, 2005), the cytogenetic analyses indicated that most patients had a normal karyotype until the clinical onset of the t-AML. This could be correct or the sample size could have been inadequate to detect an emerging preleukemic clone.

Gene expression changes

In recent years there has been great interest in applying genomic technologies to improve the diagnosis and treatment of patients with leukemia. Microarrays allow thousands of mRNA transcripts to be assessed simultaneously and powerful algorithms have been developed to allow efficient mining of the resulting data. Software has also been developed to allow pathways and networks to be identified from the gene expression patterns. While most efforts have focused on identifying the biological characteristics of the leukemias and developing a more accurate classification of leukemic sub-types, this approach has the potential to identify new and potentially specific biomarkers that will be predictive of future leukemic risk.

The initial expression studies on childhood leukemia were performed by Golub and colleagues who used gene expression arrays to develop a molecular classification of leukemia (Golub *et al.*, 1999). Using the array data, they were able to separate ALL and AML by their gene expression patterns. These initial results have been followed by others who have expanded these studies to adults and focused on using these genomic approaches to identify and classify subtypes of leukemia, compare the patterns with those seen in normal hematopoietic cells, investigate the role of certain genetic alterations, and identify patients at risk for resistance to chemotherapy or at risk for relapse following treatment (Staal *et al.*, 2003; Ross *et al.*, 2004; Willenbrock *et al.*, 2004; Andersson *et al.*, 2005; Cario *et al.*, 2005; Carroll *et al.*, 2006). Larsen and associates used expression profiling in bone marrow CD34+ hematopoietic progenitor cells to investigate gene expression patterns that characterize sub-types of t-AML (Qian *et al.*, 2002; Larson and

Le Beau, 2005). In this relatively small study, two groups were identified; leukemias with a clonal -5 or 5q- karyotype had a higher expression of genes involved in cell cycle control, checkpoints or growth, and loss of expression for the gene encoding the IFN consensus sequence-binding protein. The second group of leukemias was characterized by a down-regulation of transcription factors involved in early hematopoiesis and an overexpression of proteins involved in signaling pathways in myeloid cells (FLT3) and survival (BCL2). Somewhat surprisingly, leukemias with the -7/7q- karyotype did not cluster with those exhibiting the -5/5q- karyotype.

Downing and associates measured gene expression patterns in 360 pediatric ALL patients to determine if profiling could be used to tailor therapy for an individual patient (Yeoh *et al.*, 2002). Distinct expression profiles were identified for each of the important leukemia sub-types, including T-ALL, E2A-PBX1, BCR-ABL, TEL-AML1, MLL rearrangement, and hyperdiploid with >50 chromosomes. In addition, a new subtype was identified based upon a unique expression profile. Moreover, a unique expression profile was identified in the ALL blasts of patients with the TEL-AML1 translocation who developed t-AML suggesting that this approach may be useful to identify patients at high risk for developing a treatment-related leukemia. In a follow-up study by the same group of investigators, gene expression profiles were performed on 228 children undergoing chemotherapy with leukemogenic agents such as etoposide (Bogni *et al.*, 2006). Expression of 68 probes corresponding to 63 genes was significantly related to the risk of developing t-MDS/AML. Expression patterns for 12 of the 13 patients that developed t-MDS/AML clustered together in one group. Distinguishing genes included transcription-related oncogenes (MYB, PAX5 and PIM2), key kinases (MAP2K1 and PGK1) and the cell cycle related genes (CCNG1, CCNG2, CCND1 and CDK8). The histone HIST1H4C was also overexpressed in the t-MDS/AML children and was the only distinguishing gene that was common to both studies. One of the problems with gene expression experiments is that, because of the multiple comparisons conducted, they are highly prone to type 1 error. As a result, it is important that the results be replicated. However with verification, these results could be quite valuable in identifying new types of leukemia-related biomarkers (or combinations of biomarkers) and help identify patients as well as environmentally exposed individuals at increased risk for developing leukemia.

Proteomic approaches

Recent advances in proteomics have led to new efforts to develop diagnostic tools and biomarkers of leukemia. Much of the initial work has focused on using tools such as two-dimensional electrophoresis and mass spectrometry to profile proteins in different classes and subclasses of leukemia with a goal of improving the classification and understanding the mechanisms underlying the disease (Cui *et al.*, 2004; Te Kronnie *et al.*, 2004; Cui *et al.*, 2005; Hegedus *et al.*, 2005; Habib and Finn, 2006; Lopez-Pedrera *et al.*, 2006). In some ways, this can be seen as an extension of existing protein-based immunophenotyping that is widely used to classify leukemias (Te Kronnie *et al.*, 2004). However, this field is rapidly expanding in new directions and with the development of new techniques such as the use of aptamers and single cell proteomics which may allow new leukemia-specific biomarkers to be identified (Irish *et al.*, 2006; Shangguan *et al.*, 2006). For example, through screening sera of leukemic patients and comparing them with those found in several groups of controls, Cui and associates identified the presence of an autoantibody against a protein known as Rho GDP dissociation inhibitor 2 in 15 of 21 (71%) patients with acute leukemia and only 2 of 42 (4%) controls. Five other protein autoantibodies were also detected which were more prevalent in the leukemia patients. These may prove to be useful as biomarkers or for developing diagnostic screens.

Because of the emerging nature of this field, only now are studies appearing in the literature in which these new proteomic strategies have been applied to identify individuals at high risk for developing leukemia. In an initial study, Joo and colleagues used two-dimensional gel electrophoresis and mass spectrometry to compare plasma proteins in 50 benzene-exposed printing company workers and compared them with those of 38 controls (Joo *et al.*, 2003; Joo *et al.*, 2004). Three proteins, T cell receptor beta chain, FK506-binding protein and matrix metalloproteinase-13, were expressed only in the benzene-exposed workers. In addition, interleukin-4 receptor alpha chain and Tcell surface glycoprotein CD1b precursor were upregulated in the plasma of the exposed workers. A correlation was seen between the levels of T cell receptor beta chain and DNA damage measured in lymphocytes using the COMET assay. No association was seen between these proteins and urinary levels of t,t-muconic acid. These results suggest

that T cell receptor beta chain could be useful as a biomarker of benzene exposure and early effects.

In a more recent proteomic study conducted as part of the UCB/NCI series of studies on benzene, Vermeulen and colleagues used an array-based proteomic approach to investigate changes in protein expression patterns in workers exposed to benzene (Vermeulen *et al.*, 2005). In two sequential studies, sera from 10 benzene-exposed workers and 10 controls were analyzed using ProteinChip arrays followed by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. Three proteins were consistently downregulated in the exposed workers of both studies. Two of these were subsequently identified as platelet factor 4 and connective tissue activating peptide III. These results suggest that the lowered expression of these two proteins could potentially be used as a biomarker of benzene's early biological effects, and provide insights into the immunosuppressive effects of benzene. However, the protein changes observed differed between the Joo and the Vermeulen studies, and reinforce the need for replication and validation of biomarkers generated through these new "omics" approaches.

Biomarkers of altered structure/function

As indicated above, most cases of myelotoxicity induced by leukemogenic agents are transient and return to normal following the end of treatment or exposure. However in a minority of cases, the induced myelosuppression can persist and progress to more serious disorders including pancytopenia and, infrequently, aplastic anemia which confers a 5-15% risk of developing leukemia (Aksoy et al., 1984; Jandl, 1987; Imashuku et al., 2003). Increased risks of developing leukemia are also seen with inherited clonal hematopoietic disorders such as paroxysmal nocturnal hemoglobinuria, congenital neutropenias, and Fanconi anemia (Harris *et al.*, 1999; Freedman and Alter, 2002).

In recent years, a number of approaches including the detection of clonal hemptopoietic abnormalities have been attempted in individuals at high risk for developing leukemia. In particular, clonality assays based on X-linked polymorphisms have been tested to identify an emerging clonal population in the bone marrow. Retrospective studies have shown the presence of clonal hematopoiesis in the bone

marrow of patients who developed T-MDS following marrow transplantation (Gale *et al.*, 1996). In a more recent study, the X-inactivation based clonality assay at the human androgen receptor locus (HUMARA) was used to determine the clonality of hematopoiesis in the bone marrows of 104 women undergoing autologous bone marrow transplantation for non-Hodgkin's lymphoma (Mach-Pascual *et al.*, 1998). At the time of transplantation and after previous chemo- and/or radiation therapy, the prevalence of polyclonal hematopoiesis was 77% (80/104), that of skewed X-inactivation pattern was 20% (21/104), and the prevalence of clonal hematopoiesis was 3% (3/104). In a subgroup of 78 patients, the value of the assay for predicting t-MDS/t-AML was evaluated. At 18 months or longer after transplantation, 53 of 78 patients had persistent polyclonal hematopoiesis, 15 of 78 had a skewed X-inactivation pattern, and 10 of 78 (13.5%) either had or had previously exhibited clonal hematopoiesis. T-MDS/t-AML developed in 2 of 53 patients with polyclonal hematopoiesis, and in 4 of 10 with clonal hematopoiesis. Thus, women with clonal hematopoiesis detected in this assay were at a substantially higher risk of developing leukemia. Cytogenetic analyses were performed for approximately half of the women at various times during the study. Cytogenetic results were available for 7 of the 10 with clonal hematopoiesis. Of the 7, 5 had an abnormal cytogenetic clone, which in each case was present in over 50% of the metaphases. In contrast, only 9 of 28 (32%) of the non-MDS patients with nonclonal hematopoiesis had abnormal cytogenetics, and in only one was the abnormality present in more than 50% of the metaphases. While this assay appears to have predictive value, it has a number of limitations. It is limited to the bone marrow of selected females, those who do not have an excessive inactivation of one of their X-chromosomes relative to the other, and the assay is unable to detect clones until they comprise 10-30% of the cell population (Mach-Pascual *et al.*, 1998; Abruzzese *et al.*, 1999). Thus, the assay is unlikely to detect preleukemic clones until fairly late in the progression of the disease.

It should also be noted that abnormal hematopoiesis is not uncommon following high dose therapy and may not lead to t-MDS/t-AML even when leukemic-related cytogenetic clones are present (Laurenti *et al.*, 2002). Irons and associates have been performing detailed studies on workers previously poisoned by benzene that have persistent and ongoing bone marrow dysplasia (Irons *et al.*, 2005). A clonal expansion of

T-cell subpopulations in the bone marrow has been seen without a detectable increase in clonal cytogenetic abnormalities in the bone marrow. Surprisingly, even though significant bone marrow dysplasia is present in the bone marrows, approximately 25% of the affected patients had normal or near normal peripheral blood counts. In similar studies, Bhatia and colleagues showed that defects in hematopoiesis with marked reductions in primitive and committed progenitor cells were common in chemotherapy-treated patients prior to transplantation and that a profound reduction in primitive progenitor cells occurred after transplantation that took 3 years or more to recover (Bhatia *et al.*, 2005). The recovery of committed progenitor cells was largely complete within one year. Risk of developing t-MDS/AML seemed to be related to a reduced recovery of committed progenitor cells and telomere function, indicative to the authors of a functional defect in the primitive hematopoietic cells.

General comments of leukemia-related biomarkers:

A number of general observations and insights into the use of disease-related biomarkers can be derived from the examples presented above. First, a large number of genetic (and other changes) have been seen in the different types of leukemia. It is very unlikely that a single or even several biomarkers will be able to detect most of the many different alterations and subtypes of leukemia. To be effective, biomarkers targeting specific classes of genetic and cellular alterations will probably need to be employed. For example, it should be possible to develop effective strategies and biomarkers targeting genetic changes characteristic of leukemias induced by alkylating agents or topoisomerase II inhibitors. As a result, understanding or knowing the likely mode of action for an environmental or therapeutic agent should substantially increase the likelihood of success. This will also facilitate the integration of biomarkers with other toxicological and epidemiological information, and enhance its use in the risk assessment process.

The examples presented above indicate that in many cases disease-related biomarkers can be used to detect early changes in individuals but these are most successful in evaluating individuals known to be at high risk or previously diagnosed with a specific genetically altered form of leukemia. These techniques are particularly

useful in retrospective studies when customized primers or probes can be used. When they occur, the genetic changes can become detectable at both early and late stages in the development of the leukemic cell. Alterations occurring in the bone marrow, if detected in the blood, may not be seen until considerably later in the process. While plausible, it is not certain that alterations or changes detected in the peripheral blood lymphocytes or plasma are reflective of events occurring in the critical pluripotent stem cell or committed cells in the bone marrow.

Levels of the disease-related biomarkers do not always correlate closely with the administered dose or exposure suggesting that other susceptibility factors are involved. In addition, many of the markers can be detected in normal individuals so that their relationship to the disease and their ability to predict the progression of the disease must involve additional determining factors. Clonal variation and evolution can also occur during hematopoiesis, particularly during abnormal hematopoiesis that occurs following significant toxicity. Many of the resulting abnormal clones appear to be genetically unstable. Not surprisingly, from among the various biomarkers, the presence of leukemia-related changes would appear to confer the greatest risk for developing the disease and offer the greatest promise as a biomarker.

Biomarkers of susceptibility

Inherited polymorphisms in genes involved in xenobiotic metabolism and other cellular processes have been associated with increased risks of myelotoxicity or leukemia in numerous studies of patients or workers exposed to leukemogenic agents. In some instances, similar associations have been seen in follow-up studies by other investigators. However, in a significant number of cases, the results have either not been repeated or have not been reproducible. Consequently, it is difficult to make firm conclusions about many of the reported polymorphisms. A list of genes that have been associated with either an increased or, in some cases, a decreased risk of leukemia is shown in Table 10. Identifying consistent associations is particularly challenging because of the biological and etiological differences in infant, childhood and adult ALL and AML, the differences in ethnicity among the patients studied, and the variety of therapeutic regimens used. Several recent reviews on polymorphisms and leukemia have been published (Cheok and

Evans, 2006; Cheok *et al.*, 2006; Sinnett *et al.*, 2006), and the reader is recommended to refer to them for additional details. The following is a brief overview of some of the genetic polymorphisms that have been repeated associated with an altered risk of developing leukemia.

One of the polymorphic genes that has been repeatedly associated with an increased risk of leukemia is NAD(P)H quinone oxidoreductase 1 (NQO1), an enzyme involved with the reduction of quinones and protecting against oxidative stress. As summarized by Smith and others (Smith, 1999; Perentesis, 2001; Smith *et al.*, 2004), NQO1 activity is closely related to sequence changes in its coding region. A single nucleotide change (C to T) at position 609 results in a proline to serine substitution with near complete loss of enzyme function in that allele. Enzyme activity is completely lacking in people homozygous for this variant whereas those who are heterozygous have an intermediate level of activity. Studies have shown that this inactivating polymorphism is overrepresented in patients with t-AML (Larson *et al.*, 1999), in those with de novo AML, particularly those with translocations or an inv(16) clonal aberration (Smith *et al.*, 2001), and in infant leukemias with a 11q23 karyotype, and infants and children with the t(4;11) form of ALL (Wiemels *et al.*, 1999; Smith *et al.*, 2002). While these initial studies indicated a consistent association with a number of different leukemia types, more recent studies have been less consistent with only a few showing an association (Naoe *et al.*, 2000) and others not (Blanco *et al.*, 2002; Sirma *et al.*, 2004; Eguchi-Ishimae *et al.*, 2005; Malik *et al.*, 2006).

Reduced activity NQO1 alleles have also been reported to be more common in benzene-exposed Chinese workers with reduced blood cell counts or who had previously exhibited benzene poisoning. In the initial study by Rothman and colleagues (Rothman *et al.*, 1997), an increased risk of hematotoxicity was seen with the C609T polymorphism whereas in a later study by the same group, only another C465T polymorphism was associated with the increase in risk (Lan *et al.*, 2004). Interestingly in another Asian population, the C465T variant was seen to be strongly associated with infant leukemia, particularly the t(4;11) form of ALL (Eguchi-Ishimae *et al.*, 2005).

Thiopurine methyltransferase (TMPT) catalyzes the S-methylation of thiopurine medications such as 6-mercaptopurine and 6-thioguanine, which are commonly used to

treat ALL. As summarized from (Cheok and Evans, 2006), the TMPT pathway is the primary mechanism for inactivation of thiopurines in hematopoietic tissues. The link between TMPT polymorphisms and mercaptopurine toxicity has been extensively investigated and studies have shown a strong relationship between TMPT deficiency polymorphisms and hematopoietic toxicity. Three variant alleles are responsible for >95% of the cases with low or intermediate TMPT activity. Patients homozygous or heterozygous for the low activity alleles are inefficient at detoxifying the mercaptopurines and accumulate high concentrations in their hematopoietic tissues. If their doses are not modified, they are at high risk for severe hematopoietic toxicity. Inherited TMPT deficiency has also been associated with a higher risk of t-AML, particularly in ALL patients treated with topoisomerase II inhibitors (Thomsen *et al.*, 1999; Gadner *et al.*, 2006). It has been postulated that the increased risk may be due to an interference of 6-thioguanine or methylated 6-mercaptopurine with DNA repair after DNA damage has been induced by other chemotherapeutic agents.

Proper folate metabolism is necessary for the maintaining the integrity of DNA. Depleted folic acid levels increase plasma homocysteine levels and can lead to a disruption in DNA methylation (Smith *et al.*, 2004). In addition, depletion can lead to uracil incorporation into DNA, diminished DNA repair capacity, and increased DNA strand breakage and chromosomal damage (Robien and Ulrich, 2003). The folate-metabolizing enzyme, 5,10-methylenetetrahydrofolate reductase (MTHFR) directs 5,10-methylenetetrahydrofolate towards methione synthesis at the expense of DNA synthesis. The MTHFR polymorphisms, the C677T and the A1298C, are associated with reduced enzyme activity and enhance the flux of folate towards DNA synthesis and repair processes (Smith *et al.*, 2004). Three case control studies have reported that children and adults with the variant forms of MTHFR have a reduced risk for ALL (Skibola *et al.*, 1999; Wiemels *et al.*, 2001; Robien and Ulrich, 2003). Of note, no association has been seen for myeloid leukemias. Polymorphisms in other folate-metabolizing genes such as thymidylate synthase and serine hydroxymethylase transferase have also been reported to reduce the risk of developing ALL (Smith *et al.*, 2005a). Interestingly, in one study, the protective effect of the MTHFR polymorphisms on ALL was only seen in Canadian children born prior to 1996, a time before folic acid supplementation was recommended

for pregnancy (Krajinovic *et al.*, 2004). In an Australian study, folate supplementation during pregnancy has also been reported to have a significant protective effect against childhood ALL (Thompson *et al.*, 2001).

Defective mismatch repair is often manifested by microsatellite instability in the cancer cells. Microsatellite instability is frequently seen in leukemias, particularly in t-AML where it has been reported to occur in ~50% of the cases (Karran *et al.*, 2003). In contrast, less than 5% of de novo leukemias exhibit microsatellite instability. While the basis for this instability is still unknown, one factor that may contribute are deficiencies in DNA mismatch repair enzymes. In a recent report, the hMSH2 mismatch repair variant was shown to be significantly overrepresented in t-AML patients that had previously been treated with 0^6-guanine-forming alkylating agents including cyclophosphamide and procarbazine, as compared with controls (Worrillow *et al.*, 2003). In addition, polymorphisms in several genes involved in DNA double-stand break repair (WRN, TP53 and BRCA2) were recently reported to be associated with a decrease in white blood cell counts in benzene-exposed workers (Shen *et al.*, 2006).

Numerous xenobiotic metabolizing enzymes (Cheok *et al.*, 2006) as well as several cytokines (Lan *et al.*, 2005) have been associated with increased risks of myelotoxicity or leukemia. However in these cases, the reports are generally limited to a small number of studies or somewhat conflicting results. While risks associated with most polymorphisms are modest in magnitude (generally ~2-fold), substantially higher risks have been seen for individuals who have more than one at-risk genotype or phenotype. For example, benzene-exposed individuals who were rapid CYP2E1 metabolizers and had the C609T slow variant for NQO1 had a 7.6-fold increased risk of benzene poisoning as compared to exposed individuals with the slow metabolizer phenotype who had one or two of the wild type NQO1 alleles (Rothman *et al.*, 1997). Individuals with combinations of at-risk genotypes have also been reported to have a substantially increased risk of ALL (Sinnett *et al.*, 2006).

While the studies to date have focused on the genotypes of the patients and exposed workers, it is possible, especially for infant and childhood leukemias, that the maternal genotype may also contribute to the child's susceptibility. Sinnett and

colleagues present some suggestive evidence that the parents' genotypes can also play a role in a child's risk of developing ALL (Sinnett *et al.*, 2006).

In vitro and animal models

For most of the biomarkers described in the previous sections, numerous in vitro and animal studies have been performed to provide a more thorough understanding of the effect being monitored and to establish a foundation for the use of the biomarker. For example, extensive studies have been conducted using in vitro and animal models to understand the metabolism of various leukemogens, and these have provided a justification for the use of various metabolites as biomarkers. Similarly, most biomarkers of effect have counterparts in animals, and studies have been performed to determine the utility of a particular marker to monitor toxicity and for its potential for use as a biomarker in humans.

Animal models have been widely used to evaluate the potential of chemicals to cause cancer and leukemia. While valuable, they have significant limitations, and are widely considered to be less than ideal. For example, while most human leukemia-inducing agents induce leukemia in rodent models, the types of leukemia induced originate from different hematopoietic lineages (Eastmond, 1997). Treatment-related leukemias in humans are predominantly myeloid in origin whereas the leukemias induced by the same agents in rodents almost always originate in lymphoid cells. For this reason as well as others, there are often significant reservations about the use of these animal models to predict leukemia in humans.

In recent years, transgenic animals have also been extensively used to investigate the properties and role of specific genes in living organisms. Genetic modifications have been performed on mice in order to study the function of many of the genes involved in hematopoiesis and implicated in leukemogenesis. In addition to normal genes, mouse models have been developed to provide a more complete understanding of the function of the various fusion genes formed by chromosome translocations in leukemic cells. Mouse models have been developed for the TEL-AML1, AML1-ETO, MLL-AF9, BCR-ABL, and PML-RARα fusion genes as well as others (Janz *et al.*, 2003). For some such as those expressing the BCR-ABL or PML-RARα genes, the mice develop the

characteristic leukemia over the course of their lives. For others such as those with TEL-AML1 or a conditionally expressed AML1-ETO gene, the transgenic animals exhibit various alterations in hematopoiesis but do not normally develop leukemia (Higuchi *et al.*, 2002; Janz *et al.*, 2003; Tsuzuki *et al.*, 2004; Fischer *et al.*, 2005). The altered preleukemic phenotypes seen in these mice exhibit features seen in the human disease and may serve as useful models for leukemias in children (and adults) that exhibit the associated translocations (Tsuzuki *et al.*, 2004). Interestingly, when the mice conditionally expressing the AML1-ETO fusion gene were treated with N-ethyl-N-nitrosourea, a potent alkylating agent, a significant portion of the mice developed a granulocytic sarcoma/AML that exhibited characteristics of human AML with the t(8;21) (Yuan *et al.*, 2001; Higuchi *et al.*, 2002). The treated transgenic mice and their littermates also developed thymic T cell lymphomas that were believed to reflect the normal tumorigenic response of mice treated with ENU.

These transgenic mouse results are consistent with human observations, and indicate that leukemia in these mouse models occurs in at least two stages – one occurring early in life and a second which triggers the onset of the leukemia. These results also indicate that it should be feasible to develop mouse models for other specific leukemia sub-types, particularly those affecting children, and that these models could be used to investigate the potential contribution of infectious agents, environmental chemicals, and various host factors in the etiology of the relevant childhood leukemias.

Application of biomarker information to risk assessment

As evidenced in the previous sections, biomarkers have been used extensively to provide information about leukemia-related risks associated with exposure to a series of therapeutic and environmental agents. Information on an agent's mode of action is becoming increasingly important in the risk assessment process. As a result, there is also increasing recognition of the value and contributions that biomarker information can make at each stage in the process. Biomarkers can play a valuable role in identifying potential toxicants or carcinogens, confirming the mode of action, providing a more reliable assessment of exposure and internal dose, and in more accurately assessing the relationship between dose and response.

The use of biomarkers can also play a critical role in the final stages of the decision-making process, particularly when evaluating the risks of an agent for which there is limited evidence of effects in humans. For example, biomarker data played an important and possibly pivotal role in the decision by IARC to list ethylene oxide as a known human carcinogen (Group 1) (IARC, 1994). In evaluating the evidence, the IARC working group concluded that there was sufficient evidence of ethylene oxide's carcinogenicity in animals but only limited evidence from human epidemiological studies. However, in making the overall evaluation, the group considered additional supporting evidence including information that ethylene oxide is a powerful mutagen across all phylogenetic levels, and that it induced persistent dose-related increases in the frequency of chromosomal aberrations and sister chromatid exchange in peripheral lymphocytes, micronuclei in bone-marrow cells of exposed workers, and a dose-related increase in the frequency of hemoglobin adducts in exposed humans and rodents. In this case, the human biomarker data provided a crucial intermediate link between the mechanistic and toxicological data obtained from animals and similar, although less convincing, observations in humans. In the future, it is likely that biomarker data will be increasingly relied upon to fill data gaps and confirm predictions generated by other in silico, in vitro and animal-based methods.

A recent report has been published describing how non-tumor data including biomarker information can be integrated the risk assessment process (Albertini *et al.*, 2003a). The article provides an insightful overview of the topic as well as case studies on 1,3-butadiene, vinyl chloride and benzene which illustrate the potential application of non-tumor and biomarker data in assessing the risks associated with these agents. The reader is recommended to refer to this article for a more thorough and systematic discussion of the use of biomarkers in risk assessment in general. This section will focus more specifically on issues specifically associated with the use of biomarkers to assess the risk of leukemia in children.

A thorough assessment of the risk of childhood leukemia requires that a number of issues and challenges be considered. Some of these are common to all risk assessments but others are quite specific and unique to childhood leukemia. Outlined

below are a number of key points that should be considered in assessing potential childhood leukemia-associated risks.

- Childhood leukemia is an uncommon disease that originates through a multistage process involving a series of genetic changes in hematopoietic stem cells. Some leukemia-associated genetic changes are rare whereas others are quite common. The detection of these changes in normal individuals may indicate a substantially elevated risk of developing leukemia whereas the risk associated with others such as a tandem duplication of the MLL gene may only be significant when other alterations have occurred. This highlights the importance of understanding the background incidence and significance of the leukemia-related biomarker being considered.
- Many different pathways and genetic alterations are involved in pediatric leukemias. Both general and leukemia-specific biomarkers exist for monitoring the major pathways. However, given the diversity, a comprehensive monitoring of even the major types changes involved in the development of leukemia is likely to prove challenging.
- While the incidence of specific subtypes may vary, overall, the types and subtypes of leukemias induced in children and adults are very similar.
- Most agents inducing leukemia in adults also appear to be capable of inducing similar types of leukemia in children.
- Biomarkers of exposure and effect, particularly leukemia-related biomarkers, that have been developed and validated in adults should be relevant for monitoring similar changes in children.
- The types of leukemia induced by xenobiotics in adults and children have similar characteristics. Children appear to be more susceptible to the leukemogenic effects of ionizing radiation but do not appear to exhibit increased susceptibility to chemotherapeutic drugs, and may be less susceptible. However, comparisons between adults and children are difficult for a number of reasons including the rarity of the disease and differences in the therapeutic regimens typically used for children and adults.

- In general, the acute myeloid leukemias induced in adults and children by alkylating agents and topoisomerase II inhibitors exhibit substantially different characteristics that allow them to be differentiated. The characteristics of AML induced by ionizing radiation are similar to those of the alkylating agents, although the range of subtypes induced is broader.
- While ALL represents a common type of leukemia in children and adults exposed to ionizing radiation, it is only infrequently induced by alkylating agents, and topoisomerase II inhibitors.
- The genetic alterations involved in the development of ALL and AML in children appear to occur in at least two stages. For many childhood leukemias, the initiating event appears to be a translocation involving leukemia-related genes that occurs prior to birth. Hyperdiploidy is another early leukemogenic event that can occur prenatally. After birth, additional events must occur for the disease to be manifest.
- For most childhood ALL and infant AML, the initiating events involved in the formation of the initial translocations occur prenatally and originate from DNA double strand breaks followed by aberrant repair through non-homologous end joining. As a result, DNA damage resulting from normal DNA metabolic processing, exposure to endogenous reactive species, or exposure to exogenous xenobiotics may contribute to the alterations seen.
- Postnatally, the critical changes in many childhood ALL are postulated to involve a delayed exposure to infection in an inadequately stimulated immune system. This then, leads to an expansion in the number of previously mutated pre-leukemic cells and increases the likelihood that the second critical mutation will occur. This suggests that in conjunction with exposure to infectious agents, exposure to immunomodulating drugs and chemicals could play a role in the etiology of childhood ALL. Exposure to exogenous DNA damaging agents associated with additional mutations and chromosomal alterations is also likely to contribute, particularly in the development of childhood AML.
- Leukemia-related translocations are present in healthy children at a frequency 100-times higher than the incidence of the corresponding leukemia. Unaffected

translocation-bearing children would likely represent a group at substantially higher risk of developing leukemia.

- Prenatal exposure to high doses of radiation is generally accepted as being sufficient to induce leukemia. This means that for a thorough assessment of childhood leukemia risks, exposures to the mother during pregnancy need to be considered as well as postnatal exposures to the child. However, the lack of a reported increase in exposed children born to pregnant mothers treated with chemotherapeutic agents suggests that the associated risks are modest in magnitude.
- Hematopoiesis occurs in different tissues and organs at various stages of fetal and embryonic development. This indicates that critical pre-natal events, including tissue-specific bioactivation, may take place in the yolk sac, liver, and lymphoid tissues in addition to the bone marrow.
- Postnatal exposure to ionizing radiation, alkylating agents, and topoisomerase II inhibitors has been shown to induce leukemias in children. Significant exposure to similar environmental or therapeutic DNA damaging agents during childhood would also be expected to be associated with an increased risk of developing leukemia.
- For most leukemogens, the increase in risk is strongly associated with cumulative dose. However, for the topoisomerase II inhibitors, the dose regimen may also play an important and modifying role. Similarly, other host factors such as nutrition as well as polymorphisms in xenobiotic metabolizing, DNA synthetic, or DNA repair genes also appear to influence the risk of developing leukemia.

<u>Examples of potential uses of biomarkers in assessing risk</u>

In practice, risk assessment decisions frequently involve one of two general scenarios, one involving an observed cluster of diseases in specific population, and the second evaluating the potential risk of a future disease in a population. In both of these scenarios, biomarker information can be quite valuable for identifying, confirming and assessing the likely risks. In most cases, this information is used in a qualitative fashion or to provide information about the mode of action or the shape of the dose response

curve. However, in some cases, biomarker information may also be used to provide quantitative information on risks. In the following discussion, the potential use of biomarker data to inform the risk assessment decisions under the two scenarios will be briefly illustrated.

In the first scenario, a hypothetical cluster of childhood leukemia has been reported and the risk assessor is one of a team asked to identify the causative agent and assess the risks to other children in the community. For this hypothetical example, let us assume that most children in the cluster have the FAB M2 subtype of AML. Cytogenetic evaluations have indicated that most of the leukemic cells exhibit clonal abnormalities involving loss of all or the long arms of chromosomes 5 and 7 (-5, 5q-, -7, 7q-). The consistency of the observed leukemias and the relatively rarity of the observed cytogenetic alterations add to the weight of evidence indicating that the observed cluster is likely to represent a nonrandom increase in leukemias resulting from exposure to an alkylating or possibly an ionizing radiation-type of environmental agent. Given this information, the team of investigators could use a variety of biomarkers to help identify the causative agent and identify other children at elevated risk within the community.

In the second scenario, children and adults in a community may be exposed to a leukemogenic agent and a team including the risk assessor is asked to identify an exposure level that would confer an acceptable level of risk. In this case, the identity of the agent is known. However, the actual levels and various sources of exposure as well as intermediate outcomes of exposure may not be adequately established. For simplicity, we will assume that as in the first scenario, the agent is an alkylating agent that induces the M2 subtype of AML that is characterized by clonal aberrations involving -5, 5q-, -7 and 7q-. In this case, the use of biomarkers could be used to more accurately estimate the levels and sources of exposure. In addition, the biomarkers could be used to help estimate the leukemic risk associated with exposure.

The following examples illustrate how biomarkers could be used to provide specific types of information for one of the scenarios above: The examples are listed by type of biomarker that corresponds to the sections above. The relevant section can be referred to for specific examples.

1) Biomarkers of exposure: Personal dosimeters could be used to quantify overall exposure levels and to identify specific sources of children's exposure to chemicals or radiation.
2) Biomarkers of internal dose: The identification of chemicals in the urine or blood could be used to identify potential environmental agents or to more accurately quantify exposure to known agents.
3) Biomarkers of biologically effective dose: The measurement of DNA or protein adducts in the blood could also be used to identify species capable of binding to DNA and protein. In some cases due to their persistence, these biomarkers could also be used to estimate exposures occurring for weeks to months prior to sampling. These can also be useful in assessing the relationship between exposure, dose and response.
4) Biomarkers of early biological effect: A comparison of the frequency of genetic changes (chromosomal aberrations, micronuclei, HPRT mutations, etc.) occurring in the peripheral blood of exposed or potentially exposed children could help determine whether exposure levels had been sufficiently high to cause persistent and non-persistent alterations. Given the associations seen in recent prospective studies, in the future it may be possible to use this cytogenetic information to estimate the cancer risks associated with elevated frequencies of chromosomal aberrations and micronuclei. Probes for the loss of entire chromosomes or specific regions of chromosomes 5 and 7 could also be used to identify specific leukemia-related genetic alterations. Similarly hematological measurements could be performed to identify those with decreased blood counts.
5) Biomarkers of susceptibility: The use of genetic polymorphisms for genes involved in the metabolism of alkylating agents or the repair of DNA damage could be used to identify children who would be more susceptible to the effects of the suspected or known agent. The use of polymorphisms can also be used to give clues to help identify as-of-yet unidentified environmental agents associated with childhood leukemias.

The choice of biomarker to be employed would most likely be determined by the information available to the research team as well as the time and resources available. Other factors such as access to the study population and available experimental expertise

would also play a role. However, as is evident from both of the scenarios, the use of biomarker data can provide valuable information that can be used to inform the risk assessment process.

Conclusions

Substantial progress has been made in recent years in our understanding of the origins of childhood leukemia and in the development of leukemia-related biomarkers for both adults and children. Leukemia develops through a multi-stage process, and in many children originates from critical genetic changes that occur in utero. Subsequent changes must occur after birth for the clinically observable leukemia to develop. In light of these two different stages, a thorough evaluation of the leukemia risk factors should include exposures to the mother during pregnancy in addition to exposures to the child. Biomarkers of exposure, effect and susceptibility are allowing children with increased exposures and risks to be identified. In particular, the development of leukemia-related biomarkers has allowed the progression of the disease to be followed, and individuals at particularly high risk for developing leukemia to be identified. However, the many pathways leading to leukemia combined with its rarity and variable onset will make it difficult to detect the critical disease-related changes and identify high-risk individuals in biomarker studies of ordinary children. It is hoped that ongoing advances in genomic, proteomic and computational technologies as well as the development of new transgenic animal models will provide additional tools that will enhance future biomarker studies and facilitate the identification of at-risk children and adults.

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Table 1. Summary of the WHO classification of tumors of hematopoietic and lymphoid tissues

CHRONIC MYELOPROLIFERATIVE DISEASES
Chronic myelogenous leukemia
Chronic neutrophilic leukemia
Chronic eosinophilic leukemia/hypereosinophilic syndrome
Polycythemia vera
Chronic idiopathic myelofibrosis
Essential thrombocythemia
Chronic myeloproliferative disease, unclassifiable

MYELODYSPLASTIC/MYELOPROLIFERATIVE DISEASES
Chronic myelomonocytic leukemia
Atypical chronic myeloid leukemia
Juvenile myelomonocytic leukemia
Myelodysplastic/myeloproliferative diseases, unclassifiable

MYELODYSPLASTIC SYNDROMES
Refractory Anemia
Refractory anemia with ringed sideroblasts
Refractory cytopenia with multilineage dysplasia
Refractory anemia with excess blasts
Myelodysplastic syndrome associated with isolated del(5q) chromosome abnormality
Myelodysplastic syndrome, unclassifiable

ACUTE MYELOID LEUKEMIAS
Acute myeloid leukemia with recurrent cytogenetic abnormalities
Acute myeloid leukemia with t(8;21)(q22;q22), (AML1/ETO)
Acute myeloid leukemia with inv(16)(p13q22) or t(16;16)(p13;q22), (CBFβ/MYH11)
Acute promyelocytic leukemia (AML with t(15;17)(q22;q12), (PML/RARα) and variants
Acute myeloid leukemia with 11q23 (MLL) abnormalities

Acute myeloid leukemia with multilineage dysplasia
With prior myelodysplastic syndrome
Without prior myelodysplastic syndrome

Acute myeloid leukemia and myelodysplastic syndromes, therapy related
Alkylating agent/radiation–related type
Topoisomerase II inhibitor–related type (some may be lymphoid)

Acute myeloid leukemia, not otherwise categorized
Acute myeloid leukemia, minimally differentiated
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia

Acute monoblastic and monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma

Acute leukemia of ambiguous lineage

B-CELL NEOPLASMS
Precursor B-cell neoplasm
Precursor B lymphoblastic leukemia/lymphoma

Mature B-cell neoplasms
Chronic lymphocytic leukemia/small lymphocytic lymphoma
B-cell prolymphocytic lymphoma
Lymphoplasmacytic lymphoma
Splenic marginal zone lymphoma
Hairy cell leukemia
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT-lymphoma)
Nodal marginal zone B-cell lymphoma
Follicular lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma
Mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary effusion lymphoma
Burkitt lymphoma/leukemia

B-cell proliferations of uncertain malignant potential
Lymphomatoid granulomatosis
Post-transplant lymphoproliferative disorder, polymorphic

T-CELL AND NK-CELL NEOPLASMS
Precursor T-cell neoplasms
Precursor T lymphoblastic leukemia/lymphoma
Blastic NK cell lymphoma

Mature T-cell and NK-cell neoplasms
T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Aggressive NK-cell leukemia

Adult T-cell leukemia/lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Enteropathy-type T-cell lymphoma
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides
Sézary syndrome
Primary cutaneous anaplastic large cell lymphoma
Peripheral T-cell lymphoma, unspecified
Angioimmunoblastic T-cell lymphoma
Anaplastic large cell lymphoma

T-cell proliferation of uncertain malignant potential
Lymphomatoid papulosis

HODGKIN LYMPHOMA
Nodular lymphocyte predominant Hodgkin lymphoma
Classical Hodgkin lymphoma
Nodular sclerosis classical Hodgkin lymphoma
Lymphocyte-rich classical Hodgkin lymphoma
Mixed cellularity classical Hodgkin lymphoma
Lymphocyte-depleted classical Hodgkin lymphoma

HODGKIN LYMPHOMA
Nodular lymphocyte predominant Hodgkin lymphoma
Classical Hodgkin lymphoma
Nodular sclerosis classical Hodgkin lymphoma
Lymphocyte rich classical Hodgkin lymphoma
Mixed cellularity classical Hodgkin lymphoma
Lymphocyte-depleted classical Hodgkin lymphoma

HISTIOCYTIC AND DENDRITIC-CELL NEOPLASMS
Macrophage/histiocytic neoplasm
Histiocytic sarcoma

Dendritic cell neoplasms
Langerhans cell histiocytosis
Langerhans cell sarcoma
Interdigitating dendritic cell sarcoma/tumor
Follicular dendritic cell sarcoma/tumor
Dendritic cell sarcoma, not otherwise specified

MASTOCYTOSIS
Cutaneous mastocytosis
Indolent systemic mastocytosis

Systemic mastocytosis with associated clonal, hematological non-mast cell lineage disease
Aggressive systemic mastocytosis
Mast cell leukemia
Mast cell sarcoma
Extracutaneous mastocytoma

Modified from WHO (2001)

Table 2. Simplified classification of the major types of leukemias

Type	Preferred name	Abbreviation	FAB type
Acute	Acute myelogenous leukemia	AML	
	with minimal differentiation		M0
	without maturation		M1
	with maturation		M2
	Acute promyelocytic leukemia	APL	M3
	Acute myelomonocytic leukemia	AMML, AMMoL	M4
	Acute monoblastic leukemia	AMoL	$M5_A$, $M5_B$
	Acute erythroleukemia	AEL	M6
	Acute megakaryoblastic leukemia	AMegL	M7
	Acute lymphatic leukemia	ALL	—
	Common	cALL	L1, L2
	Acute T cell leukemia	T cell ALL	L1, L2
	Acute B cell leukemia	B cell ALL	L3
	Pre-B cell leukemia	PBCL	—
	Acute mast cell leukemia	—	—
Chronic	Chronic myelogenous leukemia	CML	—
	Chronic myelomonocytic leukemia	CMML	—
	Chronic lymphatic leukemia	CLL	—
	Chronic B cell leukemia	B cell CLL	—
	Chronic T cell leukemia	T cell CLL	—
	Prolymphocytic leukemia	PLL	—
	Hairy cell leukemia	HCL	—

Modified from Jandl (1996)

Table 3. Estimated frequencies of specific clonal cytogenetic abnormalities among children and adults with ALL

Aberration	Affected gene	Children (%)	Adults (%)
t(12;21)	*ETV6-CBFA2*	22	2
t(4;11), t(11;19), t(1;11)	*MLL rearrangements*	6	7
t(1;19)	*E2A-PBX1*	5	3
t(9;22)	*BCR-ABL*	4	25
14q11	*TCR$\alpha\delta$*	4	6
7q35	*TCRβ*	3	2
t(8;14), t(2;8), t(8;22)	*MYC*	2	4
Hypodiploidy (< 45 chromosomes)		1	4
Hyperdiploidy (> 50 chromosomes)		25	6
Apparently random		28	41

Modified from Pui and Evans (1998).

Table 4. Frequencies of the more common clonal cytogenetic abnormalities in adult and childhood AML

Cytogenetic abnormality	Adults (n = 4257) No. (%)	Children (n = 1184) No. (%)
None (normal karyotype)	1922 (45.1)	283 (23.9)
+8	387 (9.1)	112 (9.5)
-7/7q-	356 (8.4)	62 (5.2)
-7	183 (5.0)	33 (2.8)
del(7q)	92 (2.5)	18 (1.5)
Loss of (7q)	29 (2.2)	11 (1.3)
t(15;17)(q22;q21)	325 (7.6)	117 (9.9)
-5/5q-	305 (7.2)	14 (1.2)
-5	105 (2.9)	4 (0.3)
del(5q)	146 (4.0)	9 (0.8)
Loss of (5q)	18 (1.4)	1 (0.1)
t(8;21)(q22;q22)	234 (5.5)	137 (11.6)
inv (16)(p13q22)/t(16;16)(p13;q22)	202 (4.7)	70 (5.9)
-Y	78 (4.1)	28 (3.8)
t/inv(11q23)	141 (3.3)	155 (13.1)
t(9;11)(p22;q23)	27 (2.1)	54 (6.4)
abn(12p)	33 (2.5)	NA
+21	79 (2.2)	60 (5.1)
abn (17p)	42 (2.2)	5 (2.0)
del (9q)	87 (2.1)	33 (2.8)
inv(3)(q21q26)/t(3;3)q21;q26)	85 (2.0)	0
del(11q)	12 (0.9)	11 (1.3)
t(9;22)(q34;q11)	34 (0.8)	2 (0.2)
t(6;9)(p23;q34)	29 (0.7)	12 (1.0)
Complex karyotype with ≥ 3 abn	206 (10.7)	36 (14.3)
Complex karyotype with ≥ 5 abn	374 (8.8)	31 (5.2)

Modified from Mrozek *et al.* (2004).

Table 5. Examples of genes exhibiting point mutations involved in various leukemias

Kirsten rat sarcoma viral oncogene homolog (KRAS)
Neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS)
Tumor protein p53 (TP53)
Acute myelogenous leukemia 1 gene (AML1 or RUNX or CBFA2)
Mixed lineage leukemia gene (MLL or ALL1 or HRX)
FMS-like tyrosine kinase 3 (FLT3)
Core binding factor alpha and beta (CBFα, CBFβ)
Kit oncogene (c-KIT)
GATA binding protein 1 (GATA1)
V-raf murine sarcoma viral oncogene homolog B1 (BRAF)
CCAAT/enhancer binding protein (C/EBP), alpha (CEBPA or C/EBPα)
Nucleophosmin (NPM1)
Colony stimulating factor 1 receptor (CSF1R or c-FMS)

Table 6. Examples of inherited syndromes predisposing to leukemia

Syndrome	Altered gene	Leukemia/lymphoma Risk	Cancer type
Ataxia telangiectasia	ATM	~12%	T-cell lymphoma T-ALL, T-PLL B-cell lymphoma
Bloom	BLM (RECQL3)	25%	AML, ALL, Lymphoma
Fanconi anemia	7 FANC genes	~10%	AML
Werner	WRN (RECQL2)	10%	AML
Familial platelet disorder	CBFA2 (RUNX1)	~50%	AML
Down	+21	>10-fold	AML, ALL
Li-Fraumeni	TP53	50% for all cancers	B-CLL, ALL, CML, Hodgkin's Burkitt's
Neurofibromatosis	NF1	350X increase	JMML, AML

Adapted from Segel and Lichtman (2004).

Table 7. Risk factors for childhood acute leukemia, by degree of certainty

Degree of Certainty	Acute lymphoblastic leukemia	Acute myeloid leukemia
Generally accepted risk factors	Males Age (2-5 years) High socioeconomic status Race (white>blacks) In utero X-ray exposure Postnatal radiation (therapeutic) Down syndrome Neurofibromatosis type I Bloom syndrome Schwachman syndrome Ataxia telangiectasia	Race (Hispanic) Chemotherapeutic agents (alkylating agents, topoisomerase II inhibitors) Down syndrome Fanconi anemia Neurofibromatosis type I Bloom syndrome Schwachman syndrome Familial monosomy 7 Kostmann granulocytopenia
Suggestive of increased risk	Increased birth weight Maternal history of fetal loss	Maternal alcohol consumption during pregnancy Parental and child exposure to pesticides Parental solvent exposure
Limited evidence	Parental smoking prior to or during pregnancy Parental occupational exposures Postnatal infections Diet Vitamin K prophylaxis in newborns Maternal alcohol consumption during pregnancy Electric and magnetic fields Postnatal use of chloramphenicol	Maternal marijuana use during pregnancy Indoor radon Postnatal use of chloramphenicol
Probably not associated	Ultrasound Indoor radon	

From Bhatia *et al.* (1999)

Table 8. Examples of known and likely human leukemia-inducing agents.

Agents widely recognized as human leukemia-inducing agents	
Busulfan	Chlorambucil
Methyl-CCNU	Melphalan
Treosulphan	Thio-TEPA
Etoposide	Bimolane
Benzene	Tobacco smoke
Ionizing radiation	Human T-cell lymphotrophic virus type 1
Human carcinogens with some evidence of an association with leukemia	
Vinyl chloride	Ethylene oxide
Formaldehyde	
Likely human leukemia-inducing agents	
BCNU	CCNU
Cisplatin	Nitrogen mustard
Procarbazine	Chlorozotocin
1,3-Butadiene	Teniposide
Adriamycin	Chloramphenicol

Table 9. General characteristics of human leukemias induced by recognized leukemia-inducing agents.

Agent	Disease[a]	FAB[b]	MDS[c]	Chromosome Abnormalities	Latency (yrs)[d]
Ionizing radiation	AML	M1-M6	+++	-7,-5,7q-,5q-	5-7
	ALL	L1-L2			8
	CML			t(9;22)	5
Alkylating agents	AML	M1,M2	+++	-7,-5,7q-,5q-	~5
Epipodophyllotoxin Topo II inhibitors	AML	M4,M5	+	t(11q23)	2-3
Dioxopiperazine Topo II inhibitors	AML	M2,M3	+	t(8;21) t(15;17)	3
Benzene	AML	M1,M2,M6 {M3, others?}	++	Mixed[e]	10-15

[a]AML- acute myeloid leukemia, ALL – acute lymphocytic leukemia, CML – chronic myelogenous leukemia

[b]Principal leukemia subtypes classified according to the French-American-British (FAB) system.

[c]MDS – myelodysplastic syndrome

[d]Approximate median latency period.

[e]Clonal chromosomal abnormalities are present but the karyotypes reported to date have been inconsistent.

Adapted from Eastmond (1997) and Eastmond *et al.* (2005).

Table 10. Examples of polymorphic enzymes and genes associated with an altered risk of leukemia or myelotoxicity in patients or highly exposed individuals.

Phase 1 xenobiotic metabolizing enzymes
NAD(P)H quinone oxidoreductase 1 (NQO1)
Cytochrome P450 monooxygenases (CYP1A1, 2E1, 3A4)
Myeloperoxidase (MPO)

Phase 2 xenobiotic metabolizing enzymes
Glutathione S-transferases (GSTM1, GSTT1)
Thiopurine methyltransferase (TPMT)

DNA synthesis or repair enzymes
Methylenetetrahydrofolate reductase (MTHFR)
DNA mismatch repair enzyme (hMSH2)
DNA base excision repair enzyme (XRCC1)

Membrane transporter
Multidrug resistance gene (MDR1)

Figure 1. Different genetic pathways in t-MDS and t-AML. Modified from Pedersen-Bjergaard *et al.* (2002)

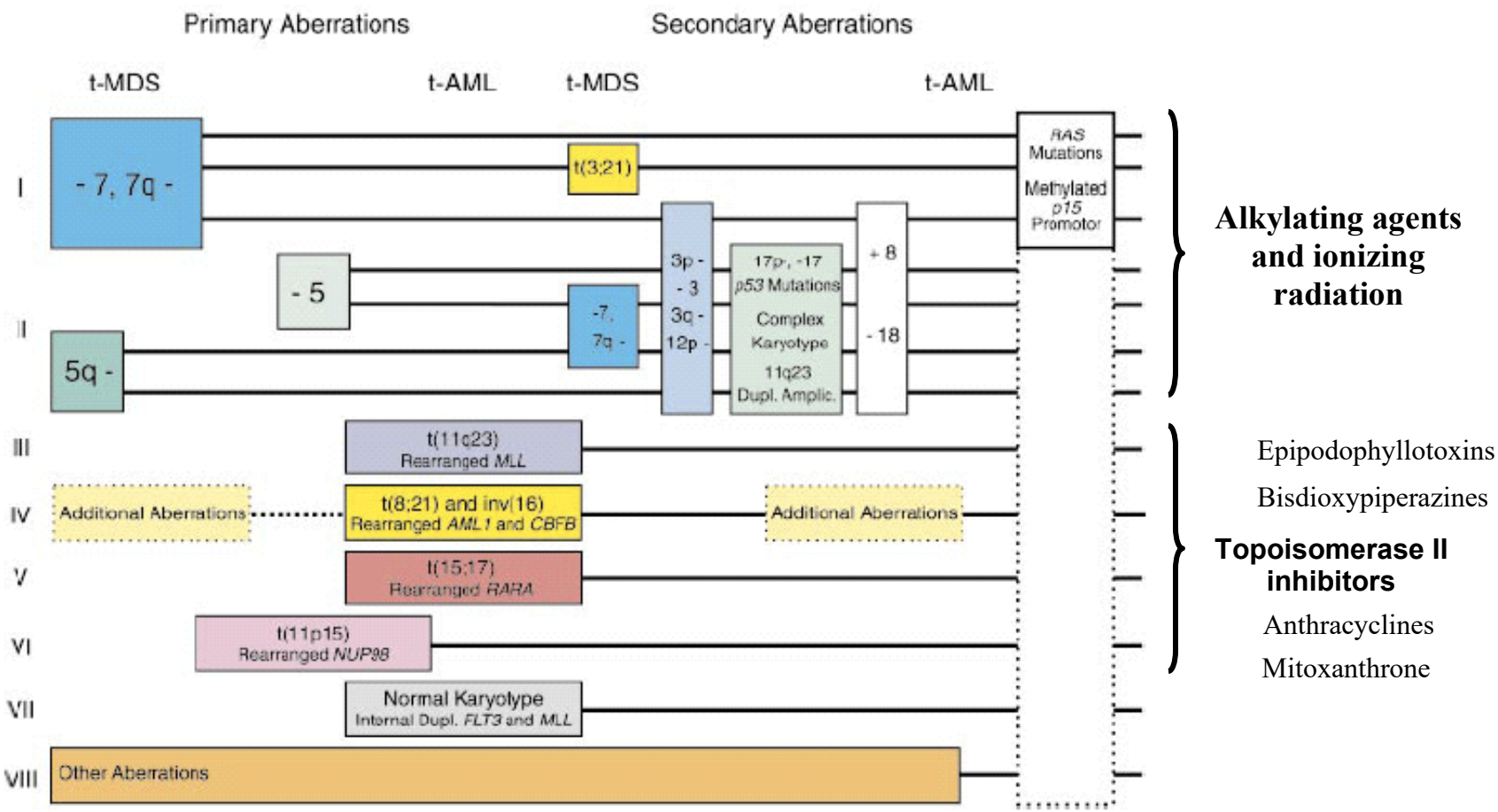

Figure 2. Biological markers in the continuum between exposure and leukemia. Modified from NRC (1987).

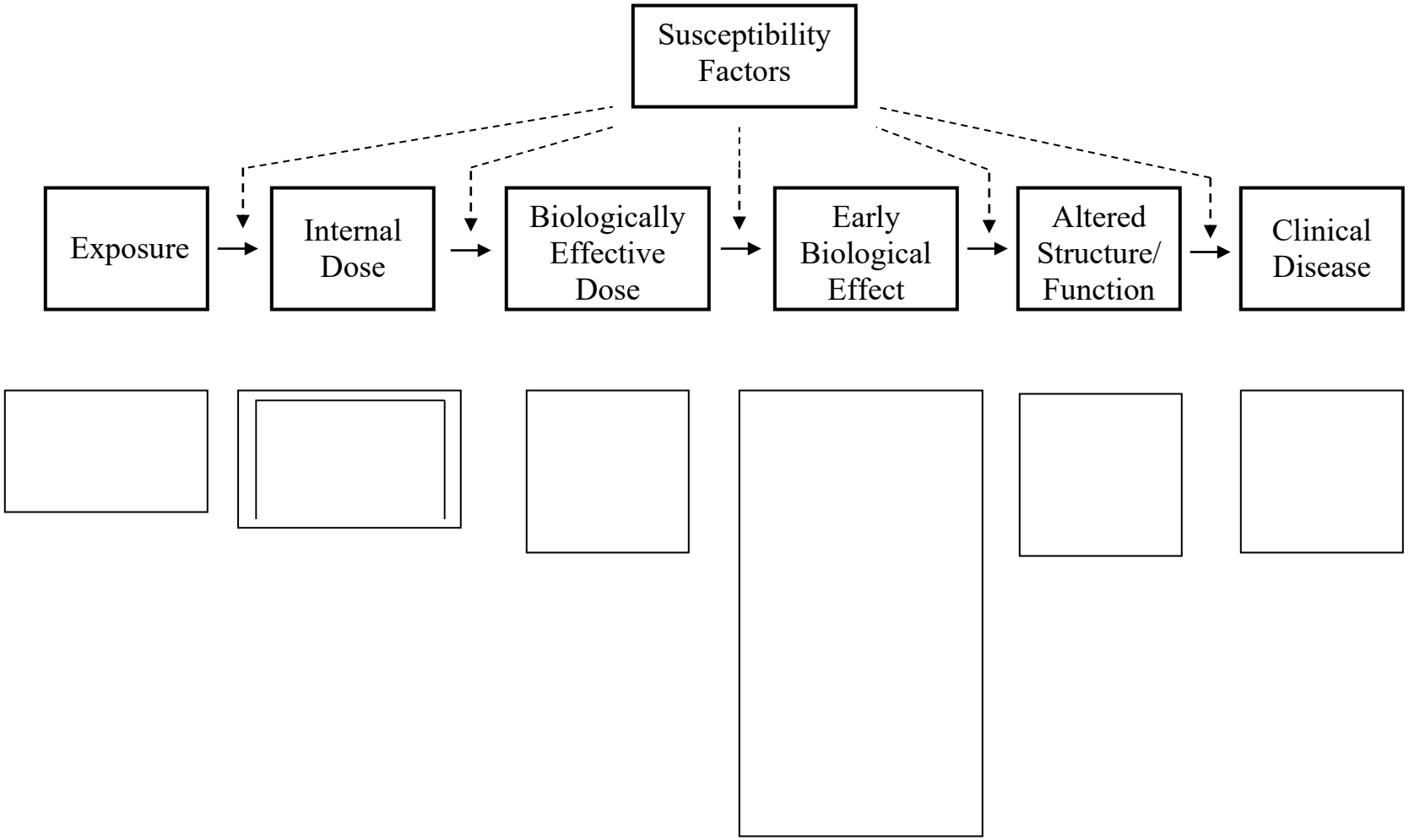

Figure 3. Refinement of exposure and dose-related biomarkers for a DNA-reactive leukemia-inducing chemical. Modified from Dahl (1990) and IPCS (1993).